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RESEARCH AND DEVELOPMENT ON INHALATION TOXICOLOGIC EVALUATION
OF RED PHOSPHORUS/BUTYL RUBBER COMBUSTION PRODUCTS

FINAL REPORT
(PHASE IV)

Prepared By

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<p>The no-measurable effect level of inhaled smoke of red phosphorus/butyl rubber (RP/BR) combustion products in male Sprague Dawley rats was evaluated in two subchronic studies. The rats were exposed for 2.25 hr/day, on 4 days/week for 13 weeks to filtered air or RP/BR aerosol, concentrations of 1.20, 0.75 and 0.30 mg/L in the first and 0.30, 0.18 and 0.05 mg/L in the second study. Aerosol mass concentration was within 3% of the target values, mass median aerodynamic diameters (MMAD) ranged from 0.40 to 0.65 µm with og's of 1.56-1.83 and the phosphorous acids in the test atmosphere varied from 71-80%. Biological endpoints examined within 24 hr of the last exposure and for selected exposure groups following an 8-week recovery period included clinical signs, body weights, pulmonary bactericidal activity and histopathology of the lungs for both studies, with pulmonary cellular responses, neurobehavioral activity and histopathology of all major organs also examined in the first (higher exposure concentration) study.</p>					
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Block 18: SUBJECT TERMS (concluded)

Exposure duration	Lowest effective dose	Pulmonary lavage
Exposure frequency	Necropsy	→ Radiolabeled bacteria
Fibrosis	Neurobehavioral activity	Red Phosphorus/Butyl Rubber
Food consumption	No measurable effect	Smoke
Histopathology	Obscurant	Subchronic exposures
→ Inhalation toxicology,	→ Phosphoric acid,	→ Terminal bronchiolar fibrosis
exposure (physiology),	Pulmonary free cells	
alveolar macrophages,		

19. ABSTRACT (continued)

→ During the first study 10.8% of the rats exposed to 1.20 mg/L died spontaneously or were necropsied in a moribund state. In addition statistically significant decreases in body weights and body weight gains were observed from weeks 1 through 13 in the 0.75 and 1.20 mg/L exposure groups. → A significant decrease in food consumption was only seen after four weeks of exposure.

→ Positive antibody titers to PVM were found in rats from the first study but no lesions characteristic of this infection were found and no deaths were due to PVM. Also such PVM-characteristic lesions are separate and distinct from the changes which were found to be exposure-related in the terminal necropsies of the studies.

→ Neurobehavioral activity parameters were not affected. Occasional statistically significant changes in pulmonary lavage parameters found in the first study after the exposures were practically all absent after recovery. Similarly, significant decreases in pulmonary bactericidal activity to inhaled 35S-K. pneumoniae at all three exposure concentrations (1.20, 0.75 and 0.30 mg/L) in the first study were completely absent after the recovery, whereas in the second study none of the exposures (0.30, 0.18 and 0.05 mg/L), including the previously positive 0.30 mg/L, produced an effect.

→ Histologically no exposure-related changes were found in tissues outside of the respiratory tract. The primary exposure-related change in the lung was terminal bronchiolar fibrosis. The microscopic examinations indicate that inhalation of RP/BR in male rats for 2.25 hr/day for 4 days/week begins to produce fibrosis after two weeks in some of the rats exposed to 0.75 mg/L and in all of those exposed to 1.2 mg/L. All animals had fibrosis at both of these concentrations after four weeks. At the end of 13 weeks, in addition to 100% of the 0.75- and 1.20 mg/L - exposure groups, less than 50% of those exposed to 0.30 mg/L and less than 25% of those that inhaled 0.18 mg/L were also affected, whereas exposure to 0.05 mg/L did not produce fibrotic changes at all. Some decrease in incidence in the lesions could be generally detected after the recovery periods, but the lesions did not disappear. Thus, the no-measurable effect level and the lowest effective dose determined by histopathological evaluation were 0.05 and 0.18 mg/L of RP/BR respectively.

→ An in vitro genetic toxicology study conducted separately from the main in vivo studies using RP/BR combustion product condensate demonstrated that this material does not cause point mutations of bacteria, primary DNA damage in rat hepatocytes, or clastogenic damage in Chinese Hamster Ovary cells under the conditions tested.

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EXECUTIVE SUMMARY

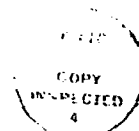
The effects of inhalation of red phosphorus butyl rubber (RP/BR) combustion products used as an obscurant smoke were evaluated in two subchronic studies with the objective of determining the no-measurable effect level and the lowest effective dose. Male Sprague Dawley rats were exposed for 2.25 hr/day, on 4 days/week for 13 weeks to filtered air or RP/BR aerosols at concentrations of 1.20, 0.75 and 0.30 mg/L in the first and 0.30, 0.18 and 0.05 mg/L in the second study.

Biological endpoints examined within 24 hr of the last exposure and for selected treatment groups following an 8-week recovery period were mortality, clinical signs, body weight, pulmonary bactericidal activity and histopathology of the lungs for both studies. In the first study, additional endpoints were food consumption, pulmonary cellular responses, neurobehavioral activity and histopathology of all major organs.

Aerosol mass concentrations were consistently within 3% of the target values. Particle size analysis showed mass median aerodynamic diameters (MMAD) of 0.49 to 0.65 μ m with σ 's of 1.56 to 1.83. Phosphorous acids in the test atmosphere ranged from 71 to 80%.

Statistically significant decreases in body weights and body weight gains were observed from weeks 1 through 13 in the 0.75- and 1.20-mg/L-exposure groups. A significant decrease in food consumption was seen in these groups four weeks after initiation of the exposures. In the first study 10.8% of the rats exposed to 1.20 mg/L of RP/BR smoke died spontaneously or were necropsied in a moribund state. Most of these animals died during the first two weeks of the exposures, and had varying degrees of congestion and small amounts of hemorrhage in the lungs. No obvious cause of death was apparent from the histopathological examination of the lungs at this timepoint. Those RP/BR-exposed (0.75 and 1.20 mg/L) animals which died during the later parts of this study had terminal bronchiolar fibrosis and erosions of the laryngeal mucosa with deposition of fibrin on the surface. These laryngeal changes were probably contributory to their death. The presence of congestion, hemorrhage, and interstitial inflammation in the lungs of 1.20-mg/L-exposed rats which died during this experiment strongly suggests that these effects were due to the RP/BR smoke. This concentration obviously produces morbidity, since, in addition to these morphologic changes, decreased body weight and food consumption were measured. No exposure-related mortalities occurred in the 0.30-mg/L-exposure groups of the first study nor in any of the RP/BR exposed (0.30, 0.18 and 0.05 mg/L) rats of the second study.

Although positive antibody titers to PVM were found in rats from the first study (including at quarantine sacrifice), no lesions characteristic of PVM infections were found in the lungs of the



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rats that died spontaneously and their deaths were not due to PVM. Also, such PVM-characteristic lesions are separate and distinct from the changes which were found to be treatment-related in animals at the terminal necropsy.

None of the neurobehavioral activity measurements conducted according to the experimental protocol showed consistent or significant changes.

Occasional statistically significant changes in pulmonary lavage parameters found after termination of the exposures in the first study were practically all absent after the recovery period, indicating that alveolar macrophages return to their normal state within 8 weeks after the exposures. This was also evident from the fact that a significant decrease in pulmonary bactericidal activity to inhaled ³⁵S- *K. pneumoniae* at all three exposure concentrations (1.20, 0.75 and 0.30 mg/L) of the first study was completely absent after the recovery period. In the second study none of the RP/BR exposures (0.30, 0.18 and 0.05 mg/L), including the previously positive 0.30 mg/L, produced an effect thus indicating that the no-measurable effect level for pulmonary bactericidal activity in male Sprague-Dawley rats under the exposure protocol used was \leq 0.30 mg/L of RP/BR aerosol.

Histologically no treatment-related changes were found in any of the tissues examined outside of the respiratory tract. The primary treatment-related change seen after termination of both subchronic studies was in the lung and was diagnosed as "terminal bronchiolar fibrosis", a lesion consisting of thickening of the alveolar walls and of the most distal portions of the terminal bronchioles at the point where they join the alveolar sacs. The use of Masson's trichrome stain showed strong evidence of collagen deposition at these sites.

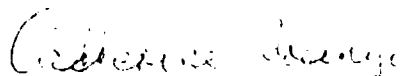
The results of the microscopic examinations of the lungs in the first study showed that inhalation of RP/BR for 2 weeks produced minimal bronchiolar fibrosis in 50% of rats exposed to 0.75 mg/L RP/BR and minimal to mild fibrosis in all of the 1.2-mg/L-exposed rats. All rats had fibrosis after 4 weeks of exposure to 0.75 or 1.2 mg/L of RP/BR. After completion of the 13-week studies 100% of the rats exposed to 0.75 mg/L RP/BR or higher and approximately 30% of rats exposed to 0.30 mg/L had terminal bronchiolar fibrosis. Severity and frequency of the lesion increased with the higher doses. In the second study there were no interim examinations. Minimal terminal bronchiolar fibrosis was found in less than 50% and 25% of the rats exposed to 0.30 and 0.18 mg/L of RP/BR, respectively. At 0.05 mg/L, the lowest dose in this study, no changes were found. In both of these studies following the eight-week recovery periods there was a decreased incidence of the lesions, but they were still present. Thus the no-measurable effect level for terminal bronchiolar fibrosis under the exposure protocol described was 0.05 mg/L of RP/BR and the lowest effective dose was 0.18 mg/L.

A separate in vitro genetic toxicology study was conducted with funds added to the contract for this purpose using condensates of RP/BR combustion products generated with the methods described in the in vivo studies. The results demonstrated that the condensate does not cause point mutations of bacteria, primary DNA damage in rat hepatocytes, or clastogenic damage in Chinese Hamster Ovary cells under the conditions tested.

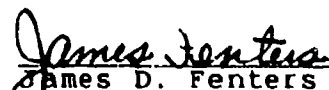
FORWORD

This report, IITRI No. LO6139, Phase IV (Final) Report describes studies conducted by the Life Sciences Department, IIT Research Institute for the Health Effects Research Division, U.S. Army Medical Bioengineering Research and Development Laboratory during the period of July 1984 through May 1986. The studies were carried out under contract No. DAMD17-82-C-2121.

Catherine Aranyi served as Principal Investigator and Study Director. Stanley Vana was responsible for the inhalation exposure facilities and the aerosol generation and monitoring throughout the studies. Jeannie Bradof and Marianna Furedi, respectively, were in charge of the pulmonary cellular response and general toxicology endpoints. The neurobehavioral activity studies were designed and conducted by Maurlene Preache. Necropsy procedures, tissue collection and preparation for histopathologic evaluation were under the supervision of Vladislava Rac and Donald E. Gordon (Consultant). Necropsy reports were prepared by Pathologists Vladislava Rac and Allan Hall, III. Histopathologic evaluation of the collected tissue samples was performed by W.D. Iverson, Consultant Pathologist from Experimental Pathology Laboratories, Inc., Herndon, VA. Robert Gibbons, Consultant Biostatistician, was responsible for statistical design and analysis. Robert Guerrero was responsible for the separately conducted in vitro genetic toxicology studies.



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QUALITY ASSURANCE STATEMENT

Numerous laboratory inspections of all critical phases of operations were conducted and reported to management during the course of the program between July 26, 1984 and January 3, 1985 for SN80 and between July 24, 1985 and January 14, 1986 by J.M. Reed. Data audits were performed February 15-18, June 4, September 19 and October 29, 1985 and January 17 to 22, 1986 by J. McPhillips. The final draft report was audited November 1 thru 4, 1986 by Josephine M. Reed. The studies were conducted according to protocol and met IITRI Life Sciences Quality Assurance criteria. Raw Data and specimens generated during the course of the studies will be retained in the IITRI Life Sciences Archives.

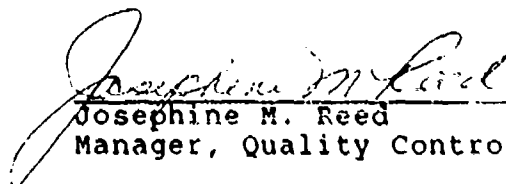

Josephine M. Reed
Manager, Quality Control

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1. INTRODUCTION

As part of an overall concern for personnel health and safety, the U.S. Army Medical Research and Development Command was seeking to evaluate the effects produced by inhalation of combustion products from red phosphorus/butyl rubber used as an obscurant smoke for troops and vehicles in tactical and training environments. Laboratory rats, exposed in chambers, were used to provide a comprehensive definition of the biological effects of red phosphorus smoke to mammalian systems under conditions which approximate the potential troop exposure. The approach to this research included range-finding acute studies to determine lethal concentrations and influence of exposure duration on mortality; repeated exposure studies to define time-concentration relationships as well as threshold levels, healing, and adaptation in biological reactions; and subchronic exposure studies with a recovery and observation period after the experimental exposure. The principal biological response criteria monitored included overt toxic signs, clinical and morphological pathology, pulmonary bactericidal activity, examination of the pulmonary lavage including alveolar macrophage functional and biochemical responses, and neurobehavioral activity. An in vivo genetic toxicology study on RP/BR combustion product condensates was added to the program during the final phase of the main in vivo studies.

2. OBJECTIVES

The objective of the Phase IV inhalation exposure studies was to evaluate the biological effects and the reversibility of the observed effects of subchronic RP/BR aerosol exposures on various biological endpoints in male Sprague Dawley rats. In Phases I through III of the studies, the aerosol exposure system was characterized for homogeneity (Phase I), range-finding acute and repeated-dose exposures were conducted (Phase II) and the combination of the effects of exposure concentration, duration and frequency on a series of biological endpoints were examined in depth after a 4-week exposure and a 2-week recovery period, respectively (Phase III). (See Section 3 of this report and for more detail Phase Report I (ADA 157686, August 1983), Phase Report II (ADA 158323, December 1983), and Phase Report III (ADA 1735493, December 1984). The primary objective of the Phase IV studies was to define the no-measurable effect level for the biological response parameters assessed to be the most sensitive indicators of dysfunction.

3. BACKGROUND: STUDY PHASES I, II, and III

3.1 PHASE I

The objective of the Phase I studies was the establishment and standardization of the inhalation exposure conditions. The inhalation exposure facility built for this project consisted of conditioned air supply and chamber air exhaust systems; inhalation exposure chambers with air flow and differential pressure controls and red phosphorus butyl rubber (RP/BR) combustion generators. Seven one cubic-meter inhalation chambers were available, five of which located in one laboratory were used for exposure to RP/BR aerosols. To assure that no contact with the experimental test atmosphere would occur, the two control chambers for exposure to filtered air were maintained in a separate room and connected to another air handling system that provided the same environmental conditions. The combined exhaust air from the five aerosol-exposure chambers was filtered through a single-housing, 30-element coalescent filter and exhausted above the roof of the building. To prevent corrosion, the filter housing was built from acid-resistant polyvinyl chloride. A pressure differential gauge installed across the filter monitored filter saturation.

The aerosol was generated by burning RP/BR extruded through specially designed hydraulic extrusion-combustion generators provided by the U.S. Army Medical Bioengineering Research and Development Laboratory through Oak Ridge National Laboratory (ORNL). The RP/BR, softened with hexane and prepackaged in stainless steel feed cylinders (billets), was also supplied by ORNL. The generator operates by exerting pressure through a hydraulic pump on the RP/BR contained in the feed cylinder. The material is forced by a piston to extrude from an orifice of the feed cylinder extending into the burn chamber of the generator and ignited by an electrically heated wire loop. The RP/BR is burned in and the combustion products are mixed with conditioned air and the aerosol is transported directly into the exposure chamber inlet port. At a constant chamber air flow rate the concentration of the aerosol is a function of the extrusion rate of the RP/BR which is controlled by the automatic hydraulic pump speed.

Throughout the studies temperature and relative humidity were monitored continuously and maintained between 24 to 27°C and 40 to 60 percent relative humidity. The aerosol was monitored for mass concentration intermittently by filter samples and continuously with photoelectric sensors, for particle size distribution with a piezoelectric microbalance-based cascade impactor, and for total phosphorus content by chemical analysis of the filter-collected aerosol samples. Oxygen concentration determined in the chambers during each exposure was consistently 21 percent. Chemical analysis of the chamber atmosphere

indicated the absence of hexane, levels of less than 10 ppb of phosphine, and variable, but relatively low, levels of carbon monoxide that could not be correlated with the RP/BR aerosol concentrations.

The objective of the Phase I studies was to evaluate spatial and temporal homogeneity of the chamber atmosphere in a three-dimensional array of points through a procedure of simultaneous sampling with cages and animal surrogates in place. For the pilot chamber, sufficient numbers of sampling points were selected to allow for characterization of spatial aerosol homogeneity within the chamber along with a series of sequential samples that were taken from a single or from multiple randomly selected fixed points to define temporal homogeneity for a period corresponding to the duration of the longest exposure. The aerosol was monitored for mass concentration, particle size and total phosphorus content at three generator settings (aerosol concentrations) replicating all tests at each generator setting three times. The ultimate objective was to reduce the variability of spatial and temporal homogeneity, with appropriate chamber modifications if necessary, to ± 20 percent of the mean of each parameter throughout the chambers and the range of concentrations tested.

Three test concentrations were selected on the following basis: the lowest operational concentration of the RP/BR generators at the 500 liter/min air flow rates used in our chambers (C_1 : 0.2-0.3 mg/L); the highest concentration that could be maintained for the 4-hr testing periods using the larger 0.75 in-diameter RP/BR billets (C_3 : approximately 1.0 mg/L) and an intermediate concentration chosen between C_1 and C_3 (C_2 : approximately 0.5 mg/L).

After standardization of the pilot chamber was completed, a single generator setting from those three evaluated for the pilot chamber was randomly selected for each of the four remaining chambers and spatial and temporal homogeneity tests were conducted in three replicate experiments for each chamber.

The statistical model used was a three-factor mixed-model analysis of variance. Concentration and location (shelf numbers 1,2,3,4 and center point) were considered to be the fixed factors, whereas replication was considered random; hence the term "mixed model". This model determines if between-location differences are nonsignificant (there is spatial homogeneity) and if differences between locations depend on concentration (there is a concentration by location interaction). In the analysis of temporal homogeneity time was substituted for location as the second factor in the design.

Shelf means and individual sampling location levels were reported in percent mean deviation units from overall chamber means. Between chamber comparisons were made by comparing overall means and examining deviations between the parameters measured in the

pilot chamber and each of the other chambers at appropriate concentrations.

The results demonstrated that the pilot chamber was spatially, as well as temporally homogeneous in terms of aerosol mass concentration and percent total phosphorus and that homogeneity was not affected by concentration.

Particle size, expressed as mass mean aerodynamic diameter (for the chamber homogeneity calculations only), appeared to increase from top to bottom in each chamber (with aerosol residence time) and also with increasing aerosol concentration. The chamber gradient within each concentration, however, was generally within the precision of the cascade impactor. In addition, the change in particle size for the entire concentration range tested was 0.3 to 0.6 μ m mass mean aerodynamic diameter which represents particles that can be inhaled and deposited in the deep lung and thus this statistical significance was not biologically meaningful in terms of inhalation and deposition of particles.

To verify that the temporal and spatial homogeneity obtained in the pilot chamber were consistent in the other four chambers, statistical analysis for each was performed. In addition, maximum location deviations in terms of worst-case shelf means were calculated for each of these chambers relative to the overall chamber means of each of the chambers. Although several of these statistical tests were significant, indicating statistical heterogeneity, the worst-case deviation for all chambers was 17 percent from the overall chamber mean. Because of the large sample size the sensitivity of the statistical evaluation was beyond what could be required with the given physical limitations of the system. Thus under the 20 percent variation limit set as our goal, the data represented adequate levels of homogeneity.

For inter-chamber comparisons, the overall means for each parameter and for each chamber were compared to the overall means of the pilot chamber for that respective concentration level. The data demonstrated that all between-chamber comparisons were within 16 percent of the pilot chamber for all measured parameters; hence it was concluded that the targeted concentration values were attained in the additional chambers.

Thus the extensive statistical analysis of the pilot chamber revealed conditions of spatial and temporal homogeneity for RP/BR aerosol mass concentration and for percent phosphorous acid levels. Although a statistically significant spatial particle size gradient was found, the variation was not significant biologically in terms of inhalation and deposition into the tracheobronchial region and the deep lung. Particle sizes were homogeneous when measured over time. Inspection of four additional chambers revealed some statistically significant differences; however, the worst-case deviations for each shelf relative to its overall chamber mean and for each chamber

relative to the pilot chamber were under the 20 percent variation limits set for the homogeneity tests on the basis of overall performance of the complex test article-generator-chamber system. Therefore it could be concluded that adequate levels of homogeneity were attained in all chambers. (For further details see Phase I Report, ADA 157686, August, 1983.)

3.2 PHASE II

The objective of Phase II was to conduct exploratory experiments using range-finding mortality studies and selected pulmonary response parameters to provide information on and the basis for selection of the exposure conditions for the definitive studies to follow in Phases III and IV.

In early exploratory experiments, male and female Sprague-Dawley rats were exposed to various concentrations of RP/BR aerosols ranging from 0.5 to 3 mg/L for durations of 1- to 4-hr. Highly significant decreases in pulmonary bactericidal activity to inhaled ³⁵S-K.pneumoniae were found in the exposed rats relative to controls after single as well as multiple exposures. The higher exposure concentrations also produced significant decreases in total cell counts in the pulmonary free cells obtained by tracheobronchial lavage from the exposed rats relative to those collected from controls.

In subsequent range-finding mortality studies male and female rats were given single 1-hr exposures to 2.00, 2.22, 2.62, 3.09 and 3.15 mg/L of RP/BR aerosols and observed for 14 days. Only the exposures to >2.62 mg/L caused deaths. The maximum mortality resulting from a single 1-hr exposure to approximately 3 mg/L of RP/BR aerosol (maximum generator capacity) was 20 to 25 percent, whereas 2.62 mg/L resulted in 6 percent deaths. A single 4-hr exposure to 0.88 mg/L, with a CxT value similar to those in the 3.09 and 3.15 mg/L 1-hr studies caused no deaths, thereby suggesting that exposure concentration is the determining factor rather than duration. These experiments demonstrated that LC50 studies with single exposures at logarithmically-spaced concentrations were not feasible in the range of aerosol concentration levels physically attainable with the generators at the air flow rates required in our inhalation exposure system.

In a follow-up multiple exposure study, rats inhaled RP/BR aerosols for 1 hr daily on 5 consecutive days at concentrations of 1.56, 1.99, 2.49 and 3.05 mg/L. Mortality rates, mean survival times, body weights and overall clinical observations were made during the 5 exposure days and for a 14-day post-exposure observation period. Necropsies were done on all animals that died during the study and on survivors on Day 19. Mortality changes for the two sexes combined ranged from 5 to 90 percent. The estimated LC50 value was 2.32 mg/L with 1.99 to 2.73 mg/L 95 percent confidence limits.

When rats received five daily 4-hr exposures to 0.35 or 0.99 mg/L of RP/BR aerosol only one died. Comparison of body weights and of clinical observations for the 1- and 4-hr studies showed markedly adverse effects after the 1-hr and practically negligible ones after the 4-hr exposures. Thus the greater influence of exposure concentration on toxicity over duration was again demonstrated.

Based on the results of these initial range-finding experiments, follow-up studies were conducted on male and female rats using four daily exposures to 0.50 mg/L of RP/BR aerosol and comparing 1.0 and 3.5-hr exposure durations. Pulmonary free cell parameters and structural changes, as evaluated by gross necropsy and histopathology, were tested immediately following the last exposure and after a 14-day recovery period. In an additional study, rats were exposed to 0.50 mg/L of RP/BR for 3.5-hr daily 4 days per week for 4 weeks. Gross pathologic observations were made after the last exposure and 2 weeks following the last exposure. All studies included daily clinical observations and regular body weight determinations.

No deaths occurred in any of the studies and generally no treatment-related clinical observations were seen. Statistical analysis of body weights showed that exposure duration was not a significant factor. When body weight changes were analyzed over the entire period of exposure and recovery, the 4-day as well as 4-week exposures produced weight decreases in male and female rats relative to controls that did not return to the normal control level within the recovery period.

Exposure duration did not have a significant effect on the pulmonary free cell parameters. Total and differential cell counts in the pulmonary lavage were not affected. Cellular ATP levels in the lavaged cells were significantly decreased when examined immediately after the fourth exposure; however, recovery was complete after 14 days.

No compound-related gross pathologic lesions were observed during necropsies immediately following the last exposure or after a 14-day recovery period in either the 4-day or the 4-week exposure group.

Microscopic examination of the tissues immediately following the 4-day exposure and after a 2-week recovery period did not reveal any treatment-related changes in kidneys, trachea and nasal turbinates. However, lungs of several animals had focal areas of interstitial reaction with alveolar macrophages which was considered to be potentially treatment-related. These animals, however, also had moderate to marked lymphoid hyperplasia of pulmonary lymph nodes which suggested that an infectious agent may have produced the pulmonary lesions. (For further details see Phase II Report, ADA 158323, December, 1983.)

3.3. PHASE III

The objectives of the repeated exposure studies of Phase III were to evaluate the interactive effects of exposure concentration, duration and frequency conditions to select the most sensitive biological response parameters for the subsequent subchronic studies of Phase IV. To accomplish these objectives, an experimental design using response surface modeling was applied. This statistical approach allowed us to examine multiple biological response parameters under a large number of experimental conditions and to select the most appropriate combinations of these factors for testing in the final experimental design. The experimental conditions included various combinations of exposure concentrations, durations and frequencies used over a four-week exposure period. The experimental endpoints tested immediately after the last exposure and after a two-week recovery period included pulmonary cellular responses, neurobehavioral activity, genetic toxicology, clinical and morphological pathology, as well as standard toxicologic observations (body weights, food consumption and clinical observations).

The investigation was divided into three main studies. The first study was to define the experimental conditions in terms of combinations of exposure concentrations, durations and frequencies that produce maximal effects in the biological response parameters selected for the project. Our statistical approach made it possible to work with a low subject number ($n=4$) for this initial study as necessitated by the complex experimental design. This design consisted of four concentrations (0, 0.40, 0.75 and 1.00 mg/L), two durations (1 and 3.5 hr) and three frequencies. The frequencies examined were exposures on two consecutive days (F1), four consecutive days (F2), or two days separated by two days of rest (F3). Maximally stressed controls inhaling filtered air for 3.5 hr/day on four consecutive days/week were used with all exposure combinations. The total exposure period was four weeks with biological assays conducted immediately after the last exposure and for two concentrations (high dose and control), one frequency (F2) and one duration (3.5 hr) tested also after a two-week recovery period. Thus animals exposed to the most stressful conditions were used in the recovery study.

With a sample size of four animals this design obviously is inadequate to test the four-way interaction of concentration by duration by frequency by recovery. This sample size is adequate, however, for tests of main effects and two-way interactions. For example, the main effect of concentration has a relative sample size of 24 animals per dosage group and control (since the design is completely balanced). The concentration by frequency and concentration by duration interactions also have adequate sample sizes. In light of this, the selected sample size in this first study yields more than adequate statistical power for an initial

characterization of the response surface involving main effects and all possible two-way interactions. Further studies using more specific experimental conditions had appropriately increased sample sizes.

The results of this first study showed relatively few compound-related effects and those that were observed, in general, also exhibited complete recovery. Although there were exceptions to this rule, given the large number of parameters tested, these effects could have occurred by chance. Furthermore, when statistically significant interactions did occur, main effects were, in general, absent. Conversely, strong main effects only occurred for parameters which did not have interactions. These findings further point to the fact that these observations were spurious, and thus it could be concluded that duration and frequency did not appear to produce major changes in the effects of exposure concentration.

Based on these results, more specific conditions with increased sample size were selected for the subsequent studies. In the second and third extended studies with male and female rats, respectively, more detailed examination of dose response relations were made for a single duration and frequency combination. Since the outcome of the first study showed that frequency and duration did not affect results significantly, exposure on four consecutive days per week (F2) was selected to explore the "worst case" situation and 2.25 hr, the mean between the previously tested 1 and 3.5 hr was chosen as the single duration. The RP/BR exposure concentrations of 0.75, 1.00 and 1.20 mg/L were used in the study with male rats and this range was lowered to 0.40, 0.75 and 1.0 mg/L for the study with female rats. The increased sample size of $n=15$ used in these two studies allowed for direct estimates of the recovery effect (i.e. concentration by recovery interaction) and also for obtaining multivariate test statistics in addition to the previously reported univariate results.

Aerosol exposure monitoring data indicate that the target concentrations were well maintained at each exposure level throughout each of the three studies. Mean RP/BR mass concentrations were consistently within 4% of the target value when measured gravimetrically and within 3% of the required concentration when determined using the light scattering photosensor. Standard deviations of the daily mean concentrations were below $\pm 8\%$ in every instance when calculated for either sampling method. Excellent agreement between the gravimetric and light scattering methods were demonstrated with variations in mean daily concentration between each method being approximately equivalent in magnitude to the deviations due to actual concentration fluctuation measured within each method. The particle size data indicate excellent aerosol stability throughout the exposures with the mean ranging from 0.44 to 0.64 μm and mean og from 1.66 to 1.97. Phosphorous acid levels ranged from 61 to 74%. The variation from sample to sample is generally

thought to include the inherent errors due to sampling and chemical analysis in addition to the actual fluctuation in phosphorous acid levels in the chambers.

During the exposure period, wheezing and labored breathing were observed in male rats exposed to the high concentration level. Decreased body weights, body weight gains and reduced food consumption, seen in male rats only, at all concentration levels during the exposures, returned to normal after the recovery period. Although an overall mortality of 12.1% was observed in male rats exposed to the high concentration level (1.2 mg/L) this was due to a 70-minute concentration overrun to 1.65 mg/L in one of the chambers on the first day of the exposures. The 5.2% mortality value observed in a second chamber reflects the effect of the exposure more realistically. In female rats only a single death was observed during the entire study and this 0.8% mortality occurred in the medium (0.75 mg/L) concentration level.

While many statistically significant main effects and concentration by recovery interactions were determined for individual response parameters in clinical pathology, only a small percentage were deemed to be biologically significant; the remainder were judged to be biologically insignificant due to their small change in absolute value relative to the control value, the associated standard deviation and their absolute value being within or close to the published normal range for the particular parameter measured. Among the prominent biological effects of RP/BR aerosols on hematology values were, therefore, decreased WBC counts in the male rats immediately after termination in the 0.75- and 1.0-mg/L-treatment groups, and their return to within-normal-limits at the end of the recovery period. Increased lymphocytes were seen in the 0.75-mg/L-treated female rats at both time periods in addition to a general and minimal trend of increased lymphocytes in all female RP/BR-exposed animals.

A greater number of clinical chemistry endpoints showed statistically significant changes. Decreased cholesterol and BUN values were seen in all RP/BR-exposed male rats at termination of the exposures with a return of values to within-normal-limits at recovery sacrifice for cholesterol, but not for BUN in the 1.0 mg/L treatment group. Treatment-induced decreases in cholesterol and triglyceride levels were seen in all RP/BR-treated females at termination. Only female rats receiving 1.0 mg/L RP/BR showed significantly decreased cholesterol and triglyceride levels after the recovery period, whereas rats receiving 0.75 or 1.0 mg/L showed decreased BUN levels at the same time point. The clinical chemistry data for both sexes suggest a forthcoming and total recovery in that the dose-response effect of treatment has diminished at recovery. A longer recovery period may have allowed for the return-to-normal values of all individual parameters.

Statistical evaluation of the pulmonary response data showed significant interactions and main effects in various categories for which subsequently individual post hoc comparisons were made. A review of the data demonstrates that in vivo bactericidal activity of alveolar macrophages (AM) to inhaled ³⁵S-K. pneumoniae and the percentage of macrophages in the cellular lavage were not affected by the exposures in either of the sexes, and phagocytosis was unaffected in female rats. Total cell counts were significantly increased in the pulmonary lavage from female rats immediately after exposure to 0.75 or 1.0 mg/L, while differential counts remained unaffected. Since 97 to 99% of the cells were macrophages, this indicates an increased number of AM in the lungs following exposures. After the recovery period the counts were no longer different from controls. The lack of increase in total cell counts for the exposed male rats was attributed to some high outlier values in the controls.

There were significant increases in cellular ATP levels of male and female rats immediately after the last exposure at all concentrations tested, except for the females exposed to 1.0 mg/L. After recovery only part of these values remained elevated. Although the pattern of these changes is not entirely clear, the general increase in cellular ATP levels indicates an increased energy supply that may be responsible for the unimpaired phagocytic and bactericidal activity observed immediately after these exposures. The most consistent finding in AM of male and female rats from all treatment groups was decreased activity of the plasma membrane-associated ectoenzyme 5'-nucleotidase (5'-ND). In addition, alkaline phosphodiesterase (ADP1) activities were decreased in AM of male rats after recovery. A decrease in 5'-ND activity is considered to be a marker for a change in the resident peritoneal macrophage (PM) population in mice, while decreased activity of both 5'-ND and ADP1 in PM has been associated with enhanced in vitro anti-tumor and antiviral activity. Although, to our knowledge, such changes have not been interpreted yet in a similar fashion for AM in rats, these data suggest that there may be a change in AM population induced by exposure to RP/BR and that these cells may have been primed for activation or activated as evidenced also by the significant increases in cellular ATP levels.

A significant increase in the protein level of the pulmonary lavage fluid of rats of both sexes after exposures to the high concentrations (1.2 and 1.0 mg/L for males and females respectively), indicating pulmonary capillary fragility, exhibited complete recovery in males.

Of the neurobehavioral parameters, locomotor activity was significantly affected by exposure to RP/BR aerosols. Male rats showed increased motor activity at all concentrations and incomplete recovery after two weeks at some concentrations. In females there was a trend toward increased activity but no evidence of effects after the recovery period. None of the

other behavioral endpoints were altered by the exposures in a consistent fashion.

A micronucleus analysis was performed on bone marrow polychromatic erythrocytes and normochromatic erythrocytes and on circulating red blood cells of female rats exposed for two or four weeks to filtered air or to 1.0 mg/L of RP/BR-smoke and after a two-week recovery period following four weeks of exposures. The results showed a significant clastogenic response in both bone marrow and RBC's of female rats that were exposed for two weeks to the RP/BR aerosol. However, no effects were found after four weeks of exposures or after a two-week recovery period following the four week exposures. Thus these results indicate that RP/BR aerosol is a weak clastogen for female Sprague-Dawley rats. The negative results found after four weeks of exposures and after the two-week recovery period suggest that the rats recruit biochemical pathways to detoxify and clear the genotoxic fractions and that under the exposure regimen used, an adaptation is in effect after four weeks of exposures.

Histopathologically no treatment-related changes were seen in tissue outside the respiratory tract in any of the studies. Changes were seen in the lung, where the primary lesion was terminal bronchiolar fibrosis which first became evident when the rats were exposed to 0.40 mg/L of aerosol for 3.5 hr/day for four consecutive days. The lesion increased in incidence and severity with increased concentrations and length of exposure and did not exhibit recovery. The use of Masson's trichrome special stain confirmed that part, but not all of the thickening of the terminal bronchioles and associated alveoli was due to fibrosis - the formation of the new collagen fibers in excess of what would normally be present.

When male rats were exposed to 0.75, 1.0 or >1.2 mg/L and female rats inhaled 0.40, 0.75, or 1.0 mg/L of RP/BR aerosols for 2.25 hr per day, on four consecutive days per week for four weeks, all, except those exposed to 0.40 mg/L, had minimal to mild to moderate terminal bronchiolar fibrosis after both the end of the exposure and recovery periods. As the thickening which comprised this lesion became more severe, increased amounts of collagen were present in these areas as evidenced by Masson's trichrome stain. A treatment-related increase in peribronchiolar eosinophilic infiltrate appeared to regress during the recovery period.

An additional study which compared rats from the Madison, WI and Indianapolis, IN breeding colonies of Harlan-Sprague-Dawley, Inc. was conducted in order to choose the most appropriate animals for the upcoming subchronic exposures. Based on the observations made in these studies and the advisability of working with PVM-free animals in an inhalation exposure, the rats from the Indianapolis breeding colony were selected for the subchronic exposures. (For further details see Phase III Report, December, 1984.)

3.4 IN VITRO GENETIC TOXICOLOGY TESTING

Within the time frame of the main Phase IV in vivo studies and with additional funds added to the contract for this purpose, a separate in vitro study was conducted to determine the genotoxic properties of the condensates of RP/BR combustion products. The organic and water soluble fractions from RP/BR aerosol generated with the same methods described in the main in vivo studies were collected and the combined fractions were tested for genotoxic properties in vitro in three short-term bioassays. The RP/BR condensate was evaluated for its ability to cause point mutations in bacteria with the Ames plate incorporation assay, for its ability to cause primary DNA damage in the form of unscheduled DNA synthesis (UDS) in primary rat hepatocytes, and to cause clastogenic damage in the form of chromosome aberrations in Chinese Hamster Ovary cells (CHO).

The RP/BR aerosol extract was prepared by collecting the condensate from 9000 liters of both filtered air and of 1 mg/L aerosol RP/BR in liquid oxygen-cooled Dewar traps. The organic fractions were extracted with methylene chloride from both samples and the residues then solubilized in DMSO. The aqueous fractions were vacuum concentrated and then the organic and aqueous fractions were combined. The air control sample was used as the solvent control in the in vitro assays. Since the RP/BR condensate was 95% phosphoric acid, phosphoric acid pH controls were included for each dose to correct for any genotoxic effects caused by pH alone.

The Ames tests and UDS test results were both unequivocally negative and the results were not influenced by low pH conditions. The chromosome aberration assay gave positive dose responses with the RP/BR condensate; however, when the results were corrected for pH effects, the clastogen damage due to the condensate was not significant. Therefore, the pH gradient alone caused the false positive dose response.

Thus based on the results of these studies, RP/BR condensate does not cause point mutations of bacteria, primary DNA damage in rat hepatocytes, or clastogenic damage in CHO cells under the conditions tested. The reports on these assays are included in toto in Appendix B.

4. MATERIALS AND METHODS

4.1. ANIMALS

Male Sprague-Dawley rats, 3 to 4 weeks-old were obtained from the Indianapolis, IN breeding facility of Harlan/Sprague-Dawley, Inc., for both studies of Phase IV. Purina Certified Rodent Chow 5002 and water were available to the rats ad libitum except during the exposures.

The animals were observed daily during a 14-day quarantine period. Prior to assignment to treatment groups all rats were subjected to physical examination and specimens were examined for pathogenic bacteria, molds, yeasts, Mycoplasma pulmonis and endoparasites. Serum samples from ten rats were sent to Microbiological Associates, Bethesda, MD, to obtain virologic antibody titers for Kilham rat virus, Toolan H-1, pneumonia virus of mice (PVM), Sendai, rat coronavirus and sialodacryoadenitis virus.

The rats were housed individually in stainless steel inhalation cage compartments measuring 18.4 x 16.5 x 15.9 cm. Twenty-four cage units, each containing four compartments, were attached to each rack housing a total of 96 rats. When attached to the racks the cages were equipped with an automatic drinking water distribution system and were suspended over excrement pans. For exposure the cages were removed from the racks and the rats are moved in their cages into the inhalation chambers. The animals were transferred weekly to clean cages and deoiled absorbing cage boards placed on the excrement pans were changed three times per week.

The holding rooms in which the test animals were housed during non-exposure periods were monitored each working day for temperature and relative humidity (RH). The mean and standard deviation for temperature and percent RH were $22 \pm 1.4^{\circ}\text{C}$ and $43 \pm 14\%$ RH. The animal rooms were maintained on 12 hr light/dark cycle.

Animals were randomized to treatment groups using a constrained random process, stratified by weight, such that all groups were comparable in pretest body weight, but assignment of individual animals to groups was random. Each test animal was identified with a unique number by an ear tag.

4.2. GENERATION AND MONITORING OF THE TEST ATMOSPHERE

4.2.1. Red Phosphorus/Butyl Rubber (RP/BR)

The test article, hexane-softened RP/BR, was supplied by the Sponsor through ORNL in tightly capped 0.75 inch-diameter and 4.5 inch-long stainless steel feed cylinders (billets) and stored at room temperature. A record of the test article was maintained which included date of receipt, identification numbers of each cylinder, and the date and study number for which it was used.

4.2.2. Inhalation Exposure Facility

The rats were exposed to the test atmosphere in seven identical 1-m³-sized stainless steel inhalation chambers operating at an airflow rate of 500 L/min. The five chambers used for exposure to RP/BR aerosol and the two filtered air control chambers were located in separate rooms.

The inhalation exposure chambers were equipped with air flow and differential pressure controls, conditioned air supply and chamber air exhaust systems, red phosphorus/butyl rubber extrusion-combustion generators and various aerosol monitoring systems.

Supply air to the inhalation exposure laboratory was conditioned by passing through particulate prefilters, charcoal filters and an air conditioning unit. Automatically controlled heating and humidifying units built into the supply air system maintained the air temperature and RH at the specified ranges of 24 to 27°C and 40 to 60 percent, respectively. Prior to entering the exposure chambers the conditioned air was further filtered through a fiberglass coarse filter, a HEPA filter and a charcoal bed.

The combined exhaust air from the exposure chambers was filtered through a single-housing, 30-element coalescent filter and exhausted above the roof of the building. To prevent acid corrosion the filter housing was built of polyvinyl chloride. The control chamber exhaust was independent from that of the experimental test chambers to avoid potential contamination from the RP/BR aerosol. The negative chamber pressures and exhaust filter loading were monitored using differential pressure gauges. Chamber airflow rates were determined by measuring the pressure differential across a calibrated in-line orifice meter.

The aerosol was generated using specially-designed hydraulic extrusion-combustion generators provided by the Sponsor through ORNL. The generator operated by exerting hydraulic pressure on a drive piston which compacted the RP/BR into a delivery cylinder, forcing the material to extrude from a smaller diameter orifice

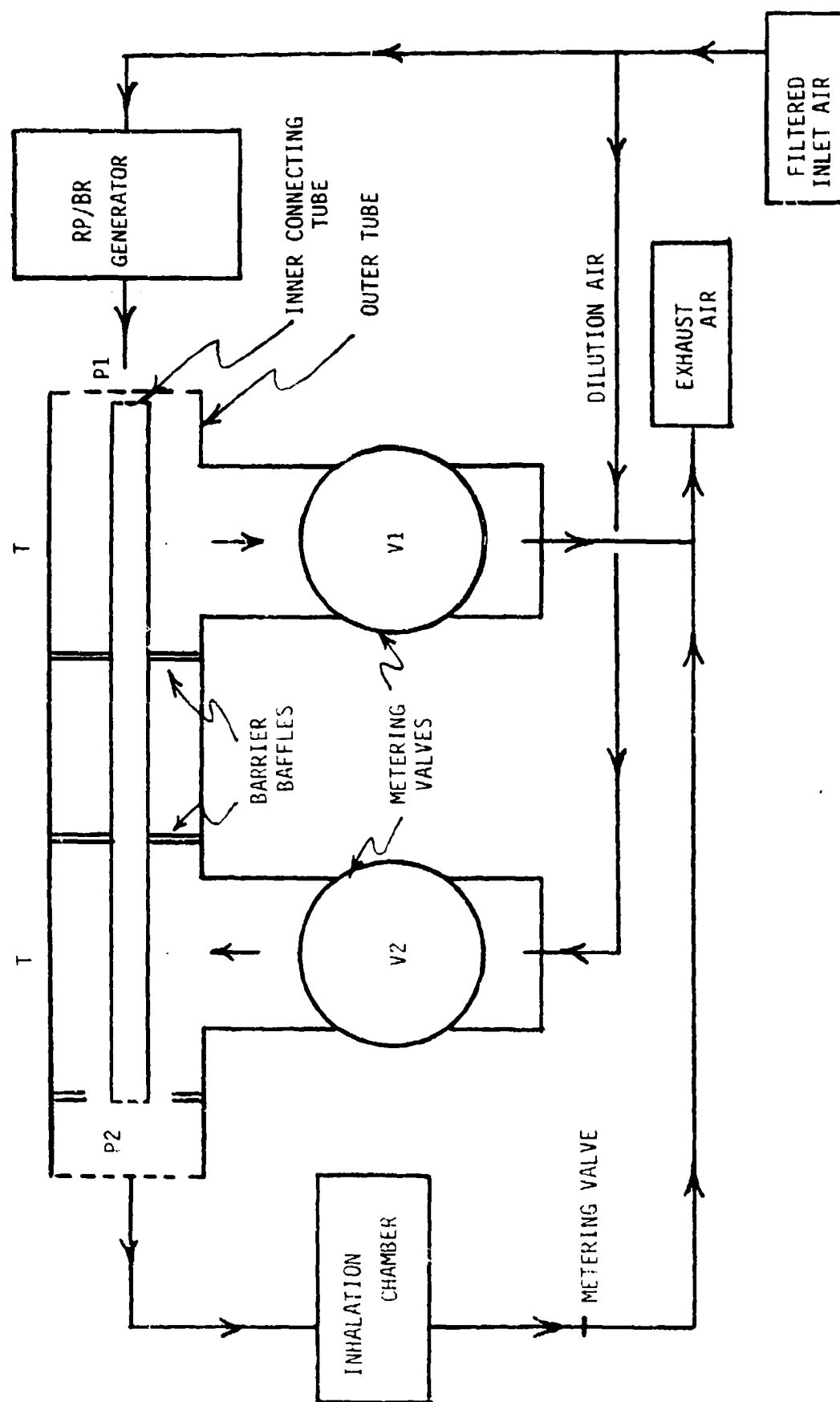
into a burn chamber where it was ignited. The aerosol, resulting from the combustion process, was transported either directly into the exposure chamber inlet, or, to achieve lower concentrations, through an aerosol dilution system. At a constant chamber airflow rate the aerosol mass concentration was a function of the extrusion rate of the RP/BR, which was controlled using a precision hydraulic metering pump.

To attain the lower concentrations (0.05, 0.18 and 0.30 mg/L) used in the second subchronic study, an aerosol dilution system was used. A schematic diagram of the design is shown in Figure 1. The system consisted of a slip stream section, for controlled removal of a portion of the original high-concentration generator effluent, and a dilution section which provides the necessary make-up air. The two sections were connected by a smaller diameter inner tube. The original aerosol-laden carrier stream entered the system at P1 and was reduced in volume by directing a portion of the aerosol flow to the exhaust through valve V1. Filtered dilution air entered the dilution section through V2. The pressure at P2 was slightly less than at P1, so that the remaining aerosol carrier stream was aspirated through the inner connecting tube and dynamically diluted in the next section. The required dilution ratio was achieved by balancing the flow rate of the aerosol through the inner tube with that of the filtered air through the dilution section. Aging of the aerosols was not a problem as the original undiluted aerosol flow rates and velocities were maintained throughout the system.

4.2.3. Aerosol Monitoring

The RP/BR aerosol mass concentration was determined three times in each chamber during the 2.25 hr exposure periods by gravimetric filter collection. In addition, the aerosol concentration levels were continuously monitored in real-time using light-scattering photosensors equipped with strip chart display of the amplifier signal outputs. An integrated average of the photosensor reading was recorded simultaneously with the gravimetric filter sample collection. Aerosol particle size was determined with a Quartz Crystal Microbalance-based cascade impactor (QCM). The particle size was monitored in each chamber once on each exposure day. Determination of total phosphorus was conducted by spectrophotometric analysis of one solubilized filter-collected sample per chamber per exposure week. All collected filters were recorded and stored in sealed containers

FIGURE 1. SCHEMATIC DIAGRAM OF AEROSOL DILUTION SYSTEM



until submitted for chemical analysis. Oxygen levels, supply air temperature and RH were monitored daily. These inhalation exposure facilities and aerosol monitoring methods have been described in more detail in the Phase I Report of this project.

4.3. BIOLOGICAL ENDPOINT ASSAYS

In the following sections the experimental methodology is described for all assays. Information on the number of rats used for each assay is provided in the subsequent section on "Experimental Design".

4.3.1. Pulmonary Response Parameters

4.3.1.1. Pulmonary Bactericidal Activity to ³⁵S-K. pneumoniae

Aerosols of ³⁵S-Klebsiella pneumoniae disseminated with a Retec X-70 disposable nebulizer were used for the bactericidal activity assay, using a method previously described for mice (Aranyi et al., 1983) and adapted for rats. Radiolabeled K. pneumoniae were grown in a medium in which the sulfate requirement of the bacteria was provided by [³⁵S] sodium sulfate. Before aerosolization, the bacteria were washed repeatedly and centrifuged for removal of unattached radiolabel. Bacterial counts were determined in a Petroff-Hausser counting chamber by dark-field microscopy and by the culture plate technique. Radioactive counts were measured in a Mark III Liquid Scintillation System (Tracor Inc.).

The radioactive bacterial aerosol exposure chamber, installed in a glove box consisted of an 87-liter main compartment (adequate for exposure of 30 rats) that was accessible through appropriate airlocks through which the animals were moved. An additional small airlock provided entry into the glove box for the nebulizers and impingers. To prevent radioactive and pathogenic exposure hazard to laboratory personnel, all air exhausted from the chamber was passed first through an absolute filter placed within the glove box and subsequently through a HEPA filter located on the outside.

Pulmonary bactericidal activity was determined in the lungs of the individual animals from both exposed and control groups that simultaneously inhaled aerosols of the viable radiolabeled bacteria. The ratio of the viable bacterial counts to the radioactive counts in each animal's lungs provided the rate at which bacteria were destroyed 3 hr after infection. Thus,

$$\% \text{ Bactericidal activity} = \left(1 - \frac{R_3}{K_0} \right) 100$$

where R_3 is the ratio of bacterial to radioactive counts in the lungs of individual rats at 3 hr, and K_0 is an average determined from the same ratios in the lungs of rats killed immediately after inhaling the bacteria.

Thus some of the rats designated for the bactericidal activity assay are killed immediately after the bacterial challenge for determination of the K_0 ratio, i.e. the ratio of viable bacterial counts to the radioactive counts at time 0. Unlike the animals that are held for 3 hr (and used in the calculation of R_3), the 0-hr animals are only used as a factor in the calculations and therefore are not included in the computation and statistical evaluation of percent bactericidal activity. In addition, in a few cases, contamination on the bacterial plates may prevent evaluation of the viable counts and calculation of bactericidal activity for a given animal. Thus the n which appears in the tables of the "Results" section is not always the same as the number of rats designated for bactericidal activity in the following "Experimental Design" section.

4.3.1.2. Pulmonary Free Cells and Lavage Fluid

Within 4 hr or 14 days after the last exposure, alveolar macrophages (AM) were obtained by tracheobronchial lavage. The rats were weighed, killed with an overdose of sodium pentobarbital by intraperitoneal injection, and the lungs were lavaged through a blunted 18 g needle inserted into an incision in the trachea with nine consecutive 6-ml infusions of warm saline. The AM were collected from the lavage fluids by centrifugation and resuspended in Hanks' balanced salt solution (HBSS). The supernatant was saved for protein determination in the lavage fluid.

Cell Counts. Total cell counts were made in a hemocytometer. For determination of the cellular distribution (i.e., percent of AM, polymorphonuclear leukocytes and lymphocytes), differential counts were made on cytocentrifuge preparations of cells fixed in methanol and stained with Wright's stain.

Cellular adenosine triphosphate (ATP) levels were determined as previously described (Aranyi et al, 1981) using a DuPont 760 Luminescence Biometer with the procedure recommended for the instrument. The assay is based on the principle that when a microsample containing ATP is injected into a suitably buffered reaction mixture of luciferase and luciferin, the peak intensity of the resulting light flash is directly proportional to the

concentration of ATP. ATP was extracted from aliquots of the cell suspension cells by dimethyl sulfoxide (DMSO). The DMSO extracts were diluted with a specially prepared 0.01 M morpholinopropane sulfonic acid (MOPS) buffer to overcome the quench effect of the high concentration of DMSO in the aqueous extract for the luciferase-luciferin reaction.

Phagocytosis. An in vitro phagocytosis assay which measures the ability of ⁵¹I rat AM to engulf ⁵¹Cr-labeled chicken red blood cells (⁵¹Cr-CRBC) was used (Smialowitz et al, 1984). AM lavaged from the lungs of exposed or control rats were adjusted to 1×10^6 AM/ml in media containing the maximum subagglutinating dilution of anti-CRBC antiserum and three 0.5 ml samples were placed in 12x75 mm sterile polypropylene tubes. ⁵¹Cr-CRBC at a 10:1 of CRBC to AM ratio are added in 50 μ l volumes to each tube. The assay tubes were placed in carrier tubes to guard against possible radioactive leakage and incubated for 1 hr on a tube rotator in a 37°C CO₂ incubator. After incubation, the tubes were centrifuged and spontaneous release counts were made on the collected supernatants. The pellets were then resuspended in lysing buffer to lyse any nonengulfed ⁵¹Cr-CRBC. The cell suspensions were centrifuged one more time, the supernatants discarded, and the AM-associated ⁵¹Cr-CRBC were counted in the pellets in a gamma counter (Tracor Inc).

Total cellular protein. For determination of total cellular protein content, aliquots of the cell suspensions were treated with 1 percent sodium deoxycholate (SDC) and assayed by the Lowry method (Lowry et al, 1951).

Lavage fluid proteins. Lavage fluids were assayed for protein with the Lowry method without SDC treatment.

Ectoenzyme Activity. Alveolar macrophage plasma membrane ectoenzyme activities were determined on aliquots of AM lysates. Approximately 2.5×10^6 AM per rat were lysed in 0.05 percent Triton X-100 and frozen until used.

- o Leucine aminopeptidase (LAP) activity was determined according to the method of Morahan (1981). The AM lysate was placed in pH 7.5 phosphate buffer and incubated for 15 min at 37°C with 10 mM leucine p-nitro-anilide substrate. The amount of p-nitroaniline released was measured at 405 nm with a spectrophotometer.
- o Alkaline phosphodiesterase (APD1). An aliquot of AM lysate was mixed with Sorenson's glycine II Buffer (pH 9.6) and incubated 30 min at 37°C to measure APD1

activity according to the method of Edelson and Gass (1981). The release of p-nitrophenol by APD1 was quantitated by measuring optical density at 400 nm in a spectrophotometer.

- o 5'-Nucleotidase (5'-N) activity was measured according to the procedure of Edelson and Duncan (1981). The AM lysate was mixed with pH₇ 9.0 Tris-HCl buffer and incubated with 25 nCi 5' [³H]-AMP/ml, 0.15 mM 5'-AMP and 6 mM p-nitrophenylphosphate for 30 minutes at 37°C. The amount of tritiated 5'-AMP hydrolyzed was measured with a liquid scintillation counter.

For calculations of ectoenzyme activities, the protein concentration of each AM lysate was determined according to the method of Bradford (Edelson and Duncan, In Methods for studying mononuclear phagocytes, Academic Press, 1981, pp. 339-343). Dilutions of AM lysates were mixed with Bio-Rad Protein Assay Dye Reagent and the optical density read at 595 nm in a spectrophotometer. Protein concentrations of AM lysates were obtained by comparing O.D. values with samples of known protein concentration using reverse linear regression.

Expression of data: Bactericidal activity was expressed as the percent of inhaled ³⁵S-K. pneumonia killed in the lungs 3 hr after challenge (% BC). Total cells counts were expressed as number of cells x 10⁶ per rat or as total cells x 10⁵ per g body weight (Totcell/g BW). The relative macrophage portion of the differential cell counts was expressed as % macrophages. Cellular protein content was reported as ug protein per 10⁵ cells. Cellular ATP content was expressed as ATP fg x 10⁶ per 10⁵ cells and as ATP fg x 10⁶ per ug protein. Phagocytosis of ⁵¹Cr-CRBC was reported as the mean radioactive count (CPM). Lavage fluid protein was expressed as total ug protein recovered per g body weight. The specific activity of each of the ectoenzymes ALAP, APD1 and 5'-N) was expressed as units of specific activity (SA) where SA equals nmoles substrate hydrolyzed/min/mg protein.

4.3.2. Neurobehavioral Activity

Subsets of the rats were tested behaviorally on the day of the last exposure. Separate groups of rats were evaluated for recovery of behavioral indices 8 weeks after the last exposure. The behavioral indices considered were fore- and hindlimb grip strength and locomotor activity as measured in a figure-eight maze. The tests were administered in the order listed above.

4.3.2.1. Locomotor Activity

The apparatuses for locomotor activity measurements were two figure-eight mazes (Digiscan Animal Activity Monitors, Omnitech Electronics, Columbus, OH) with an eight-channel printout counter (Datalogger 8000, Omnitech). In the figure-eight maze, activity is measured as photobeam interruptions for 8 sets of photobeam-sensor combinations spaced through the arms of the maze. Each animal was placed individually in the figure-eight maze for a period of 20 min and an interim count was printed after the first 10 min. Thus, the three activity counts analyzed were expressed as: activity for the first 10 min, activity for the second 10 min and activity for the total 20 min.

4.3.2.2. Fore- and Hindlimb Grip Strength

The apparatus and procedures for fore- and hindlimb grip strength were as described by Meyer et al (Neurobehav. Toxicol. 1, 233, 1979). Briefly, this test employed pull-push strain gauges (Chatillon, Models DPP-1.0 kg and DPP-2.5 kg, J.A. King, Greensboro, NC) to measure the grip force of the fore- and hindlimbs. The animal was placed in a trough and allowed to grip with its forepaws a triangular grasping ring which was attached to the forward-mounted strain gauge. The animal was steadily pulled by the tail away from the ring until the grip was broken. Pulling was continued until the hindlimbs grasped a T-bar mounted on a second strain gauge behind the trough, and further continued until the grip of the hindpaws on the T-bar was broken. The animal received three consecutive trials and fore- and hindlimb grip strength (grams force) was recorded from the forward and rear strain gauges. The fore- and hindlimb grip strength scores for each animal were derived by averaging the results of the three trials.

4.3.3. Standard Toxicology

4.3.3.1. Mortality, Clinical Observations, Body Weights and Food Consumption

All animals were observed daily in the morning for survival, physical appearance, behavior and any pharmacologic and/or toxicologic signs. All observations were recorded on an individual test animal basis. In addition afternoon survival checks were performed.

Each animal was weighed, using a Mettler PE 1600 balance with a special animal weighing mode, at the time of test animal selection, at the initiation of the study, weekly thereafter and prior to the last exposure. For the rats designated for recovery, the body weights were recorded at the aforementioned time points and continued weekly until study termination.

During the first study average food consumption (FC) was measured for individual rats over 24-hr periods every four weeks (Weeks 4, 8, 12, and during the recovery period at Test Weeks 16 and 20) for animals designated for general toxicology (TOX) endpoint parameters in the protocol.

4.3.3.2. Necropsy and Histopathology

Rats were killed with sodium pentobarbital and exsanguinated from the abdominal aorta, either following 13 weeks of exposure or after 8 weeks of recovery following the exposures, and necropsied under the supervision of the staff pathologist. (Interim necropsies were conducted following 2 and 4 weeks of exposure on 6 rats each of the medium and high dose and control groups in the first study only to explore the onset of pulmonary fibrosis.) The necropsy procedure included a thorough examination and dissection of the animal viscera and carcass and collection and fixation of all major tissues in 10% neutral buffered formalin (NBF). All tissues and/or organs were examined in situ before dissection from the carcass for individual examination. Tongue, trachea, lungs and pulmonary lymph nodes were removed intact. The lungs and nasal passages were infused with and subsequently submersed in NBF. Tissues were fixed not less than 48 hours prior to trimming.

Tissue trimming (wet sectioning) was performed at IITRI under the supervision and direction of the staff pathologist. Organs were trimmed to allow the largest surface area possible for examination. Each lung lobe was sectioned along its main bronchus. Both a cross section and a longitudinal section of the trachea were made. Two transverse sections of the skull through the nasal turbinates were made at the incisor's and the palatal ridge level.

For all 24 designated animals of the terminal (12) and recovery (12) groups of the first study and all animals found dead or killed in a moribund state the following tissues and/or organs were microscopically examined: larynx, trachea, pulmonary lymph nodes, each lobe of the lungs, and two levels of the nasal turbinates. In addition esophagus, heart, liver, kidneys, adrenals, duodenum, eyes, stomach, urinary bladder and testes were processed for all terminal and recovery animals from the control and the 1.2 mg/L-treatment-groups. For rats killed in the interim necropsies after 2 and 4 weeks of exposure, only the lungs were processed and examined.

For the second study the necropsy procedure included examination and collection of the following tissues from 20 rats designated for pathology: tongue, larynx, trachea, thyroids/parathyroids, lungs with respiratory lymph nodes, thymus, nasal turbinates, heart, kidneys, liver, brain, gross lesions and tissue masses.

The following tissues and/or organs were examined microscopically: Each lobe of the lungs with pulmonary lymph nodes and gross lesions were prepared for all 20 necropsied animals per treatment group. In addition, the trachea and one transverse section of the skull through the nasal turbinates (at the palatal ridge level) were examined in 10 selected animals (even numbered) per concentration and time point.

Tissues in paraffin blocks were submitted to Experimental Pathology Laboratories Inc. (EPL), Decatur, IL where hematoxylin and eosin stained sections were prepared and examined. Selected specimens from lung tissue were also examined by Masson's trichrome stain for demonstration of collagen fiber formation.

4.4. STATISTICAL METHODS

Two general statistical approaches to the analysis of these data were taken. In terms of BC activity, a three factor mixed-model analysis of variance was performed. The fixed effects in this design were concentration (with 4 levels) and recovery (with two levels) and the random factor was replication; hence the term "mixed-model". In the presence of a significant main effect of concentration or concentration by recovery interaction, post hoc comparisons were made using Dunnett's test. Per cent BC data were natural log transformed prior to analysis to better approximate the assumed normality of these data.

The same statistical approach was used for the analysis of continuous pulmonary response parameters (i.e. ATP, % macrophages, total cell counts, lavage fluid protein, phagocytosis and macrophage enzyme activities LAP, APD1, and 5'N) and neurobehavioral activity parameters (i.e. activity in first 10 minutes, second 10 minutes and total activity, and fore leg and hind leg grip strength). These data were similarly

transformed to better approximate the assumed normality of the statistical test.

In terms of the repeated body weight measurements, a so-called growth curve model (Bock, 1975) was used to examine the effect of concentration on the rate of body weight gain over time.

In the presence of a significant main effect or interaction, post hoc comparisons were conducted using Dunnett's test.

4.5. EXPERIMENTAL DESIGN

The design for the two subchronic studies was finalized in consultation between the Principal Investigator, Dr. R. Finch, COTR, Dr. M. Henry, former COTR, and Dr. R. Gibbons, IITRI's consultant biostatistician. Dr. William Iverson, consultant veterinary pathologist participated in the design of the second study.

The experimental design and the exposure conditions for the first subchronic study were developed based upon the results of the Phase III studies and included exposures for 2.25 hr/day on four consecutive days per week for a period of 13 weeks to 0.30, 0.75 and 1.20 mg/L (C_1 , C_2 and C_3) of RP/BR aerosol, or for controls exposure under the same conditions to filtered air (C_0). The experimental endpoints selected for evaluation of the effects after 13 weeks of exposure and for the C_2 , C_3 and C_0 treatment groups after an 8-week recovery period included standard toxicologic observations (body weights, food consumption, overall observations), histopathology of the lungs and other major organs, examination of the pulmonary free cells collected from the lungs by lavage, measurement of in vivo pulmonary bactericidal activity and evaluation of neurobehavioral activity parameters. The biological and statistical evaluation of these studies were expected to provide information on the no-measurable effect level for the RP/BR exposures.

The results of these studies demonstrated that, although changes in several of the parameters examined could be seen, the most consistent effects were the terminal bronchiolar fibrosis found in the lungs of all RP/BR treatment groups including the recovery groups and the significant³⁵ depression in pulmonary bactericidal activity to inhaled *S-Klebsiella pneumoniae* measured immediately after the exposures to all RP/BR levels.

A second subchronic study therefore was conducted to determine the no-measurable effect level in the most sensitive response parameters. Three aerosol concentrations, 0.05, 0.18 and 0.30 mg/L, were selected (repeating the lowest concentration from the first study as the highest in the second) to examine the fibrotic changes in the lungs and the changes in pulmonary bactericidal

activity after a 13-week exposure as well as after an 8-week recovery period. The frequency and duration of the exposures were maintained as in the first study at 2.25 hr/day on four consecutive days/week.

The specific experimental design parameters selected for the two studies are described in the following:

4.5.1. Experimental Design Parameters For Both Studies:

Animals: Male rats only

Exposure duration frequency and period: 2.25 hr/day on 4 consecutive days per week (F2 of Phase III) for a period of 13 weeks followed for selected groups by 8 weeks of recovery.

4.5.1.1. Experimental Design Parameters for the First Study

Exposure concentrations: Three RP/BR aerosol concentrations 0.30 mg/L (C_1), 0.75 mg/L (C_2), 1.20 mg/L (C_3) and filtered air (C_0).

Biological endpoints: In vivo pulmonary bactericidal activity (BC); Lavage: pulmonary free cell and lavage fluid parameters (LAV); neurobehavioral activity tests (BEH); general toxicology: clinical observations, body weights, food consumption (TOX); morphological pathology (PATH).

Number of animals: Thirty-six or 32 (recovery) rats per treatment group were assigned for BC and 24 for LAV. Twenty-four animals from each treatment group were assigned to be used jointly for BEH, food consumption (TOX) and 12 of these were randomly selected for PATH. Not all endpoints were measured in all treatment groups. (See following "Assay Schedule".) All animals under test (612 initially) were examined for the TOX endpoints of clinical observations and body weights, whereas, as stated above, food consumption was determined on 24 animals per treatment group.

Assay Schedule: Biological endpoints were tested within 24 hr after the last exposure and for selected treatment groups after an 8-week recovery period (R) as follows:

BC, LAV, TOX, PATH: C₀, C₁, C₂, C₃; C₀(R), C₂(R), C₃(R).
BEH¹: C₀, C₁, C₃, C₀(R), C₃(R).

For the TOX endpoint, food consumption was determined every four weeks and body weights were determined weekly throughout the study on all animals. In addition, interim necropsies were conducted after 2- and 4-week periods on 6 rats per group exposed to C₀, C₂, and C₃ for respiratory tract histopathology.

4.5.1.2. Experimental Design Parameters for the Second Study

Exposure concentrations: Three RP/BR aerosol concentrations 0.05 mg/L (C₁), 0.18 mg/L (C₂), 0.30 mg/L (C₃) and filtered air (C₀).

Biological endpoints: In vivo pulmonary bactericidal activity (BC) and morphological pathology (PATH)

Number of animals per treatment group: PATH: 20, BC: 42

Assay schedule: Biological endpoints were tested within 24 hr after the last exposure and also after an 8-week recovery period (R) following the last exposure.

4.5.2. Execution of the Experimental Design

Because of the labor-intensive nature of the endpoint assays for the number of rats required in the experimental design, the 13 weeks of exposures were staggered over a 14-week period for both studies. In the first study, post exposure assays were conducted on the days of the last exposures for BC, LAV and BEH, whereas PATH was done the day following the last exposures. Recovery experiments were conducted 8 weeks (56 days) after the last exposure for LAV and BEH and 57 days after the last exposure for BC and PATH.

¹ Although not included in the experimental design, due to an error in animal distribution logistics on one of the experimental endpoint assay days following the recovery period, a C₂-treatment-group (from the additional RP/BR-exposed animals that were available) was submitted to and consequently tested for BEH, thus resulting in an unplanned C₂(R) BEH group.

In the second study, half of the animals for each parameter were exposed to C_0 , C_1 , C_2 , and C_3 in the early morning and the other half in the late morning. (For explanation of this arrangement see Section 5.1. "Experimental test atmosphere".) Post exposure experiments were conducted on the days of the last exposures for BC and necropsies (PATH) were done on the day following the last exposures. Recovery experiments were conducted 8 weeks (56 days) after the last exposure for PATH and 57 days after the last exposure for BC.

In addition, to utilize project personnel in conducting all endpoint experiments most efficiently, on the week of the endpoint assays the four daily exposures were also staggered by conducting them for various treatment-groups from Monday through Thursday and Tuesday through Friday, respectively. Details of the exposure and assay dates for the different endpoints and the actual animal numbers assigned for each group were summarized in tables included in the experimental protocols filed with the study records.

5. RESULTS

5.1. THE EXPERIMENTAL TEST ATMOSPHERE

The data characterizing the experimental test atmosphere for the two subchronic studies (high and low concentration range) are summarized in Tables 1 and 2, respectively, and include overall means and standard deviations of mass concentration, particle size parameters and phosphorous acid levels for each chamber. For each exposure period, the aerosol mass concentration was determined by collecting three 20-min gravimetric filter samples with an integrated average of concurrently recorded photosensor concentration measurements. Aerosol particle size was determined daily at each concentration. Phosphorous acids were analyzed from one filter-collected sample per week for each chamber. Oxygen monitored daily in each chamber was consistently 21%. Temperature and RH of the air supply were maintained at 24-27°C and 40-60%, respectively.

The RP/BR aerosol exposures were conducted at target concentrations of 0.30, 0.75 and 1.20 mg/L in the first (Study Number 80) and at 0.05, 0.18 and 0.30 mg/L in the second (Study Number 82) subchronic study for 2.25 hr/day on four consecutive days/week (Mondays through Thursdays) for periods of thirteen weeks.

In the first study, rats were exposed to the RP/BR aerosol in five chambers. One chamber was used at the 0.30-mg/L-, and two each at the 0.75- and 1.20-mg/L levels and for the filtered-air-exposed controls. The additional chambers were needed because of the space requirements for the additional animals included according to the experimental protocol in these three treatment groups (C₂, C₃ and C₀) for the recovery studies.

The lower concentrations of 0.05, 0.18 and 0.30 mg/L of RP/BR used in the second study were produced by using an aerosol dilution system on three chambers as previously described (see Methods). In this study, recovery groups were included for all three aerosol exposure concentrations, as well as the filtered-air controls. Since, again, it was not possible to accommodate the total number of rats required at each concentration level in one chamber and since only one dilution system was available for each target concentration, it was necessary to conduct two 2.25-hr exposures daily in each chamber. The first exposure was started at approximately 8:45 A.M. and the second around 11:30 A.M. immediately following completion of the first.

Thus, means of the test atmosphere characterization data were calculated from the daily means for five chambers in the first study (one at 0.30 and two each at 0.75 and 1.20 mg/L). In the second (low concentration) study, two overall daily means were

calculated for each of the three target concentrations measured in the three chambers for the two sequential exposures.

To accommodate the experimental design defined in the protocols with their extensive labor and animal requirements for the endpoint assays, the animals were divided into appropriate subgroups and the 13-week exposures were staggered over 14-week periods. In both studies at each target concentration all days of exposure chamber operation were used in the calculation of the overall concentration means. Therefore the number of daily means for mass concentration in Tables 1 and 2 reflects the total number of days each of the chambers were used for exposures during each study.

The data shown in the tables indicate that the target concentrations were well maintained at each of the exposure levels throughout both studies. Close agreement between the gravimetric and light-scattering methods were demonstrated at all concentrations. In both studies the mean RP/BR aerosol mass concentrations determined by the gravimetric method and by the light-scattering photosensors were within 2.0 and 2.7 percent, respectively of the target values. Relative standard deviations of the daily mean concentrations were below ± 7.1 percent in every instance. The particle size data indicate aerosol stability throughout the exposures with the MMAD ranging from 0.49 to 0.65 μm , and the mean geometric standard deviations (ag) from 1.56 to 1.83. Phosphorous acid levels ranged from 71.4 to 79.5 percent throughout the two studies.

5.2. PULMONARY CELLULAR RESPONSES

Male rats were exposed to filtered air or to RP/BR aerosol at concentrations of 0.30, 0.75 or 1.20 mg/L (first study), or 0.05, 0.18 and 0.30 mg/L (second study) for 2.25 hr/day, 4 days/week for 13 weeks. Pulmonary cellular responses were measured within 24 hr after the last exposure or after an 8-week recovery period following the last exposure. (Only control, medium and high-concentration exposure groups were included in the recovery groups of the first study, whereas all groups were examined after recovery in the second study.) In the first study, the endpoints included pulmonary lavage parameters and pulmonary bactericidal activity. Thus the rats were killed, pulmonary free cells and lavage fluid were collected from their lungs by tracheobronchial lavage and a series of assays were conducted on the separated cells and the lavage fluid as described in Section 4.3.1.2. of

Table 1

**TEST ATMOSPHERE CHARACTERIZATION DATA FOR RP/BR AEROSOLS CALCULATED FOR
THE FIRST SUBCHRONIC INHALATION EXPOSURE STUDY^a**

Chamber No.		Target Conc.	Aerosol Mass Concentration, mg/L				Aerosol Particle Size						Phosphorous Acid ^b				
			Determined from:														
			Filter Samples		Photocensors		MMAD ^c		Q _g ^c								
			Mean	+ SD	N ^d		Mean	+ SD	N ^d	Mean	+ SD	N ^d					
1		0.30	0.296	0.021	58	0.30	0.01	58	0.49	0.08	55	1.83	0.20	55	71.8	6.2	14
2		0.75	0.739	0.032	59	0.74	0.03	59	0.51	0.08	58	1.79	0.20	58	71.7	4.2	14
3		1.20	1.186	0.047	58	1.15	0.03	58	0.52	0.08	58	1.77	0.17	58	71.6	6.2	14
4		0.75	0.739	0.040	52	0.73	0.03	52	0.50	0.09	52	1.78	0.21	52	74.6	4.7	13
5		1.20	1.189	0.045	52	1.16	0.03	52	0.51	0.09	52	1.77	0.18	52	74.3	3.1	13

^a Rats were exposed for 2.25 hr/day on four consecutive days per week for thirteen weeks. To accommodate the number of animals exposed to the medium and high doses two chambers were used at each of these concentrations. Because of a one-week staggering in the experimental schedule the total period of exposure chamber use was 14 weeks.

^b Total phosphorous acids in the aerosol determined after oxidation as H_3PO_4 .

^c Mass Median Aerodynamic Diameter (um) and Standard Geometric Deviation.

^d Calculated from the daily means over the entire exposure period (N days).

^e Calculated from one filter-collected aerosol sample per week over the entire exposure period (N weeks)

Table 2

TEST ATMOSPHERE CHARACTERIZATION DATA FOR RP/BR AEROSOL CALCULATED FOR
THE SECOND SUBCHRONIC INHALATION EXPOSURE STUDY^a

		Aerosol Mass Concentration, mg/L				Aerosol Particle Size				Phosphorous Acid						
		Determined from:														
Chamber No.	Target Conc.	Filter Samples			Photosensors			MMAD ^c			G ^c					
		Mean	+ SD	N ^d	Mean	+ SD	N ^d	Mean	+ SD	N ^d	Mean	+ SD	N ^e			
1	0.05	0.049	0.002	52	0.05	0.00	52	0.58	0.01	52	1.60	0.02	52	79.5	7.9	13
2	0.18	0.180	0.006	52	0.18	0.01	52	0.55	0.01	52	1.56	0.02	52	74.6	3.8	13
3	0.30	0.302	0.010	52	0.30	0.01	52	0.65	0.01	52	1.60	0.02	52	72.7	7.4	13
1	0.05	0.050	0.003	52	0.05	0.00	52	0.58	0.01	52	1.60	0.02	52	79.4	7.7	13
2	0.18	0.178	0.005	52	0.18	0.00	52	0.55	0.01	52	1.56	0.02	52	74.1	4.0	13
3	0.30	0.307	0.011	52	0.30	0.01	52	0.65	0.01	52	1.59	0.02	52	71.4	5.0	13

^a Rats were exposed for 2.25 hr/day on four consecutive days per week for thirteen weeks. To accommodate the number of animals on test Chambers Nos. 1, 2 and 3 were used twice daily for sequential 2.25-hr periods.

^b Total phosphorous acids in the aerosol determined after oxidation as H_3PO_4 .

^c Mass Median Aerodynamic Diameter (μm) and Standard Geometric Deviation.

^d Calculated from the daily means over the entire exposure period (N days).

^e Calculated from one filter-collected aerosol sample per week over the entire exposure period (N weeks).

"Materials and Methods". Other rats from each treatment group were used for determination of in vivo pulmonary bactericidal activity as described in Section 4.3.1.1. In the second study only the pulmonary bactericidal activity assay was used.

Statistical evaluation of the data of the first study showed a significant ($p < 0.0001$) multivariate treatment by recovery interaction indicating differing responses in rats examined immediately after the last exposure and in those tested after an 8-week recovery period. In other words, generally there was a recovery for effects observed initially in pulmonary cellular responses. Significant univariate interactions were found for total cells ($p < 0.04$), total cells/g body weight ($p < 0.02$), ATP/ 10^5 cells ($p < 0.03$), ATP/ μ g protein ($p < 0.001$), and the ectoenzyme activities of LAP ($p < 0.001$) and 5'N ($p < 0.001$). In addition, significant main effects of concentration were found for total cells ($p < 0.001$), % BC ($p < 0.001$), total cells/g body weight ($p < 0.003$), % macrophages ($p < 0.001$), μ g protein/ 10^5 cells ($p < 0.001$), phagocytosis ($p < 0.001$), lavage fluid protein ($p < 0.05$), and the ectoenzyme activities of LAP ($p < 0.005$) and APD1 ($p < 0.001$). Individual post hoc comparisons were made subsequently for all parameters with significant interactions or main effects. Significant differences ($p < 0.05$) obtained by post hoc comparisons of exposed to control groups are indicated in Tables 3 and 4 showing the means with associated standard deviations for all pulmonary response parameters examined immediately after the last exposure and after an 8-week recovery period, respectively.

A review of these data demonstrates a significant dose-related decrease in pulmonary bactericidal activity to inhaled ³⁵S K. pneumoniae at all three exposure concentrations (0.30, 0.75 and 1.20 mg/L) tested immediately after the final exposure (Table 3). Recovery from these effects was complete at 8 weeks after the last exposure (Table 4). The data on the pulmonary cells obtained by lavage immediately after the final exposure do not show consistent changes (Table 3). The total number of free cells recovered from the lungs (expressed as total cells $\times 10^6$ and as total cells $\times 10^3$ /g body weight) generally showed a decreasing trend with significant decreases at the 0.30 mg/L RP/BR level. The increases seen in % macrophages determined by differential counts are not considered biologically meaningful. Significant increases in cellular ATP levels, suggesting a change in biochemical metabolism, were found in the AM lavaged from the 1.20-mg/L-exposure group when expressed as ATP/cells and in the 0.30- and 1.20-mg/L-exposure groups when expressed as ATP/protein. Cellular protein levels, phagocytosis, and the protein content of the lavage fluid showed only scattered changes. There

Table 3

FIRST STUDY

EFFECTS OF EXPOSURES TO RP/BR AEROSOLS FOR 2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK FOR 13 WEEKS ON PULMONARY RESPONSE PARAMETERS OF MALE SPRAGUE-DAWLEY RATS TESTED IMMEDIATELY AFTER THE FINAL EXPOSURE (MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS))

ASSAY ^a	0.0 mg/L	0.3 mg/L	0.75 mg/L	1.2 mg/L
% DC	86.20 ± 7.19 (34)	70.96 ± 12.80 (24)*	52.90 ± 18.58 (23)*	49.66 ± 23.75 (24)*
TOT CELLS	13.06 ± 5.56 (19)	9.65 ± 3.49 (24)*	12.69 ± 1.97 (24)	13.84 ± 3.93 (24)
TOTCELL/g BW	31.66 ± 14.34 (19)	23.61 ± 9.16 (24)*	31.92 ± 5.21 (24)	36.20 ± 11.88 (24)
%MACROPHAGES	95 ± 4 (19)	96 ± 4 (24)	99 ± 2 (24)*	99 ± 2 (24)*
PROT/10 ⁵ CELL	25.65 ± 3.16 (19)	24.80 ± 3.04 (24)	22.73 ± 3.22 (24)*	23.70 ± 4.23 (24)
ATP/10 ⁵ CELL	0.85 ± 0.31 (19)	1.05 ± 0.21 (24)	0.91 ± 0.24 (24)	1.07 ± 0.34 (24)*
ATP/ug PROT	3.31 ± 1.19 (19)	4.26 ± 0.82 (24)*	4.02 ± 0.85 (24)	4.55 ± 1.21 (24)*
PHAGO [CPM]	10247 ± 2070 (15)	9816 ± 1115 (20)	9933 ± 1343 (20)	10501 ± 1628 (20)
LAVPROT/g BW	11.42 ± 2.15 (13)	9.33 ± 2.34 (21)*	11.01 ± 1.76 (24)	11.37 ± 2.98 (24)
LAP	16.98 ± 5.58 (17)	13.42 ± 4.83 (22)	10.90 ± 4.55 (24)*	9.73 ± 4.62 (24)*
APDI	4.61 ± 1.67 (18)	4.59 ± 0.99 (22)	4.92 ± 1.17 (24)	4.04 ± 1.68 (23)
5'-N	8.00 ± 2.86 (19)	6.97 ± 2.31 (21)	5.80 ± 1.95 (24)*	5.30 ± 2.10 (24)*

^a %DC: pulmonary bactericidal activity; TOT CELLS: total cells x 10⁶/rat; TOTCELLS/g BW: total cells x 10³/g body weight;
^b MACROPHAGES: from diff. cell count; PROT/10⁵ CELL: ug protein/10⁵ cells; ATP/10⁵ CELL: ATP fg x 10³/10⁵ cells; ATP/ug
 PROT: ATP fg x 10³/ug protein; PHAGO [CPM]: mean count of phagocytized Cr-CRBC; LAVPROT/g BW: ug lavage fluid protein/g body weight; LAP (leucine aminopeptidase), APDI (alkaline phosphodiesterase 1) and 5'-N (5'-nucleotidase): nmoles substrate hydrolyzed/min/mg protein

*SIGNIFICANTLY DIFFERENT (p<0.05) FROM CORRESPONDING CONTROL GROUP

Table 4

FIRST STUDY

EFFECTS OF EXPOSURES TO RP/BR AEROSOLS FOR 2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEK FOR 13 WEEKS ON PULMONARY RESPONSE PARAMETERS OF MALE SPRAGUE-DAWLEY RATS TESTED AFTER AN 8-WEEK RECOVERY PERIOD
(MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS))

ASSAY ^a	0.0 mg/L	0.75 mg/L	1.2 mg/L
Z BC	71.55 ± 10.70 (17)	66.19 ± 11.19 (19)	59.78 ± 26.91 (20)
TOT CELLS	12.40 ± 2.76 (24)	9.75 ± 3.81 (23)*	10.83 ± 2.46 (24)
TOTCELL/g BW	28.51 ± 6.90 (24)	22.88 ± 9.48 (23)*	25.35 ± 5.50 (24)
MACROPHAGES	97 ± 3 (24)	98 ± 3 (17)	99 ± 1 (24)
PROT/10 ⁵ CELL	19.25 ± 4.41 (24)	19.25 ± 4.17 (23)	19.23 ± 2.42 (24)
ATP/10 ⁵ CELL	1.05 ± 0.33 (24)	1.02 ± 0.32 (23)	0.96 ± 0.27 (24)
ATP/ug PROT	5.45 ± 0.90 (24)	5.28 ± 1.06 (23)	5.13 ± 1.45 (24)
PHAGO [CPH]	20812 ± 3459 (24)	20097 ± 1973 (23)	20764 ± 2636 (24)
LAVPROT/g BW	9.23 ± 1.45 (24)	9.62 ± 2.03 (22)	10.32 ± 2.46 (21)
LAP	17.20 ± 5.54 (23)	20.81 ± 6.63 (21)	16.42 ± 5.76 (23)
APDI	3.22 ± 1.36 (23)	4.14 ± 1.64 (22)	3.03 ± 0.99 (22)
5'-N	5.61 ± 2.75 (23)	6.81 ± 2.67 (22)	5.65 ± 2.87 (23)

^a ZBC: pulmonary bactericidal activity; TOT CELLS: total cells x 10⁶/rat; TOTCELLS/g BW: total cells x 10³/g body weight;
^b MACROPHAGES: from diff. cell count; PROT/10⁵ CELL: ug protein/10⁵ cells; ATP/10⁵ CELL: ATP fg x 10³/10⁵ cells; ATP/ug
 PROT: ATP fg x 10³/ug protein; PHAGO [CPH]: mean count of phagocytized ⁵¹Cr-CRBC; LAVPROT/g BW: ug lavage fluid protein/g body
 weight; LAP (leucine aminopeptidase), APDI (alkaline phosphodiesterase 1) and 5'-N (5'nucleotidase): nmoles substrate
 hydrolyzed/min/mg protein

*SIGNIFICANTLY DIFFERENT (p<0.05) FROM CORRESPONDING CONTROL GROUP

was a significant decrease in cellular protein levels measured after exposure to 0.75 mg/L of the aerosol. Although a significant main effect of exposure was seen in phagocytosis of ⁵¹Cr-CRBC, none of the post hoc comparisons was significant. Lavage fluid protein expressed as ug protein/g body weight was significantly decreased only at the 0.30-mg/L-RP/BR level.

There were occasional changes in the plasma membrane-associated ectoenzymes of AM obtained from RP/BR-exposed rats. Decreased activities of LAP and 5'N were observed in rats that inhaled 0.75 or 1.20 mg/L RP/BR aerosol, however, APDI activity was not affected at any of the exposure levels.

The results from the studies conducted in the recovery groups shown in Table 4 demonstrate that practically all of the RP/BR-induced changes in the pulmonary response parameters were reversible. The two significant changes in total cell counts were in a treatment group (0.75 mg/L) that did not show an effect in the tests conducted immediately after the last exposure. Thus, cessation of exposure to RP/BR for an 8-week period appears to allow resident alveolar macrophages to return to their normal state.

Statistical analysis of the bactericidal activity data obtained from the second study showed no main effects, and therefore post hoc comparisons were not applicable. The data for post-exposure and the post-recovery experiments are summarized in Table 5. It can be seen that for the 0.30 mg/L-treatment group tested immediately after the last exposure there was a considerably greater standard deviation than there was in the first study at the same exposure concentration (See Table 3). This may explain the fact that, whereas a significant decrease in bactericidal activity was observed after exposure to 0.30 mg/L RP/BR aerosol in the first study, this was not the case in the second study. Thus, the no-measurable effect level was < 0.30 mg/L as determined by pulmonary bactericidal activity, one of the two most sensitive indicators of the potentially adverse effects of RP/BR aerosol inhalation found in our studies.

5.3 NEUROBEHAVIORAL ACTIVITY

Neurobehavioral activity parameters were tested only in the first study. The only significant effect was a decrease in hindlimb grip strength measured at the end of the 8-week recovery period for the rats exposed to 0.75 mg/L (Tables 6 and 7). (This 0.75-mg/L-exposure group was not included at all for neurobehavioral activity studies in the experimental protocol and the rats were submitted for testing due to an error in logistics.) Since only

Table 5

SECOND STUDY

EFFECTS OF EXPOSURES TO RP/BR AEROSOLS FOR 2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK FOR 13 WEEKS
ON PULMONARY BACTERICIDAL ACTIVITY (%) OF MALE SPRAGUE-DAWLEY RATS TESTED
IMMEDIATELY AFTER THE FINAL EXPOSURE OR AFTER AN 8-WEEK RECOVERY PERIOD
(MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS))

TIME OF ASSAY	0.0 mg/L	0.05 mg/L	0.18 mg/L	0.30 mg/L
POST EXPOSURE	79.04 ± 11.84 (26)	82.08 ± 11.86 (28)	77.43 ± 16.55 (29)	72.40 ± 24.50 (27)
POST RECOVERY	77.91 ± 8.04 (28)	74.99 ± 13.58 (30)	72.95 ± 11.88 (30)	75.40 ± 10.07 (29)

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Table 6

FIRST STUDY

EFFECT OF EXPOSURES TO RP/BR AEROSOLS FOR 2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK FOR 13 WEEKS ON NEUROBEHAVIORAL ACTIVITY OF MALE SPRAGUE-DAWLEY RATS TESTED IMMEDIATELY AFTER THE FINAL EXPOSURE (MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS))

Endpoint Assays	0.0 mg/L	0.3 mg/L	0.75 mg/L	1.2 mg/L
Locomotor Activity^a				
Act-1st 10'	124.00 ± 33.76 (24)	121.71 ± 25.05 (24)	---- ± 0.00 (0)	125.04 ± 29.80 (24)
Act-2nd 10'	44.75 ± 18.06 (24)	42.63 ± 12.65 (24)	---- ± 0.00 (0)	45.79 ± 22.24 (24)
Act-Tot 20'	168.75 ± 48.57 (24)	164.33 ± 33.08 (24)	---- ± 0.00 (0)	170.83 ± 46.82 (24)
Grip Strength^b				
FL Grip	923.7 ± 212.9 (23)	979.7 ± 191.5 (24)	---- ± 0.0 (0)	972.2 ± 250.1 (19)
HL Grip	561.91 ± 88.26 (23)	561.42 ± 74.34 (24)	---- ± 0.00 (0)	558.11 ± 80.00 (19)

^a Activity counts in the figure-8 maze for the first 10 min of testing (Act-1st 10'); for the second 10 min of testing (Act-2nd 10'); for the total of 20 min of testing (Act-Tot 20').

^b Forelimb grip strength (g force), average of 3 trials (FL Grip); Hindlimb grip strength (g force), average of 3 trials (HL Grip).

Table 7

FIRST STUDY

EFFECT OF EXPOSURES TO RP/BR AEROSOLS FOR 2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK FOR 13 WEEKS ON NEUROBEHAVIORAL ACTIVITY OF MALE SPRAGUE-DAWLEY RATS TESTED AFTER AN 8-WEEK RECOVERY PERIOD [MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS)]

Endpoint Assays	0.0 mg/L	0.75 mg/L	1.2 mg/L
<u>Locomotor Activity</u> ^a			
Act-1st 10'	106.79 ± 32.22 (24)	109.23 ± 22.21 (22)	117.79 ± 31.09 (19)
Act-2nd 10'	45.63 ± 25.38 (24)	46.55 ± 17.13 (22)	35.89 ± 18.11 (19)
Act-Tot 20'	152.42 ± 51.85 (24)	155.77 ± 31.72 (22)	153.68 ± 46.63 (19)
<u>Grip Strength</u> ^b			
FL Grip	1366.9 ± 228.8 (24)	1331.8 ± 255.1 (24)	1308.8 ± 193.3 (21)
HL Grip	694.29 ± 112.50 (24)	617.92 ± 108.79 (24)*	690.00 ± 97.05 (21)

^a Activity counts in the figure-8 maze for the first 10 min of testing (Act-1st 10'); for the second 10 min of testing (Act-2nd 10'); for the total of 20 min of testing (Act-Tot 20').

^b Forelimb grip strength (g force), average of 3 trials (FL Grip); Hindlimb grip strength (g force), average of 3 trials (HL Grip).

*=SIGNIFICANTLY DIFFERENT (p<0.05) FROM CONTROL GROUP

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the 0.75 mg/L recovery animals were used in this non-scheduled experiment, this effect is considered questionable. In addition, the rats exposed to the higher concentration of 1.20 mg/L showed no performance decrease on the last day of the last exposure or 8 weeks later. In contrast to the evidence from 4-week exposures (Phase III Report) that locomotor activity was increased by exposure to RP/BR aerosols, male rats exposed to the test aerosols at 0.3 or 1.20 mg/L for 13 weeks showed no alterations in activity either on the day of exposure or 8 weeks later. Although no final conclusion can be drawn as to the basis for the difference between this study and those conducted previously, several factors should be mentioned. In the subchronic study, activity measures were made following, rather than prior to, the grip strength tests. It is possible that the change in order of testing interfered with finding an effect. In the subchronic study, specifically with respect to the second 10-min of activity testing, the within-group variability tended to be relatively high. However, this was not the case for the first 10-min of testing and the means across groups are comparable, suggesting that the lack of effect in the later study was not due to altered within-group variability. Other possibilities are that the effect was adapted out with the longer exposure period of the subchronic study or that the age of the rats at the time of testing activity was critical. Rats in subchronic study were older at the time of testing due to the longer exposure and recovery periods.

5.4 STANDARD TOXICOLOGY AND PATHOLOGY SYNOPSIS¹

5.4.1. First Study

5.4.1.1. Spontaneous Deaths and Moribund Sacrifices

When male rats were exposed to RP/BR aerosols as previously described, a total of 22 rats died spontaneously (18) or were killed in a moribund state (4) prior to their scheduled terminations during the first study (Table 8). At the 1.20-mg/L-exposure level, 10.8% (19/176) died with one death each after 1, 2, 11, 30, 35 and 45 exposures, two deaths after 6, 7 and 8 (moribund) exposures and 7 deaths after 5 exposures (2 of the 7 were moribund). In addition, one control rat died after 40

¹ A detailed Pathology report from EPL with histopathologic incidence tables prepared by Dr. W. Iverson is included in the Appendix.

TABLE 8

DISTRIBUTION OF DEATHS DURING THE
FIRST SUBCHRONIC STUDY¹

Exposure Conc. mg/L	Number of Rats ²	Total Number of Deaths	Number of Deaths
			Following (Number of Exposures)
1.20	176	19	1(1,2,11,30,35,45); 2(6,7,8); 7(5)
0.75	176	1	1(R)
0.30	84	0	
0	176	2	1(40); 1(R)

¹There were 4 consecutive exposure days each week over a period of 13 weeks followed by an 8-week recovery period (R).

²Total number exposed to that dose and designated for all biologic endpoint determinations (including recovery animals). For details see Experimental Design in Section 4.5.1.1. .

exposures. During the recovery period, one rat from the 0.75-mg/L-exposure group and one from the filtered air control group died. Thus most, but not all, of these 22 mortalities were from the high-dose group and many occurred within the first two weeks of the exposures. Tissues from these animals were submitted after necropsy to EPL for histopathologic examination.

From these twenty-two animals, those sixteen that died during the first four weeks of the exposures prior to the second interim necropsy had varying degrees of congestion and small amounts of hemorrhage in the lung tissue. Other lesions seen were similar to those found in the interim-terminated animals (see Section 5.4.1.3.1.). No obvious cause of death was apparent from examination of the lungs from these animals.

From the six animals which died during the later parts of the study, four that had been exposed to RP/BR (three to 1.2 and one to 0.75 mg/L) had terminal bronchiolar fibrosis. The three high-concentration (1.20 mg/L) animals had mild to severe erosions of the laryngeal mucosa with deposition of fibrin on the surface. These laryngeal changes were probably contributory to the death of these three animals (see also quoted statement of the study pathologist in the following).

Since one of the ten rats tested from the quarantine animals of this study had positive serum antibody titer to PVM, serum samples were submitted to Microbiological Associates from five rats (two of those found in a moribund state after 8 exposures to 1.20 mg/L and listed above and three rats from the 0.75-mg/L-exposure group that were killed for this purpose without any clinical signs of disease). All five animals had highly positive serum antibody titers to PVM.

At this point the question arose if the PVM infection could have contributed to the mortalities, or its presence cause more severe morphological changes, or if it could have been a contributing factor to the fibrosis caused by the RP/BR exposures (see histopathology observations described in following sections). Dr. W. Iverson, Veterinary Pathologist in charge of evaluation of the histopathology in these studies provided the following opinion: "Pneumonia virus of mice (PVM) is a pneumovirus first described in 1940. Recent studies have shown that it is of low transmissibility, even among animals housed together, and extremely labile in the environment. Most laboratory rodents, including rats, are susceptible. The characteristic lesions associated with the virus are an acute vasculitis which develops into patchy interstitial pneumonia. Perivascular and peribronchiolar accumulations of lymphocytes are also present. Fibrosis at the terminal bronchiole, which is the primary effect of RP/BR exposures, has not been reported with PVM infections. Therefore, the lesions produced by PVM infection are separate and distinct from those which were treatment-related (Smith, et al., 1984; Vogtsberger, et al., 1982; Hunt, et al., 1978).

The combination of lesions produced by PVM was also not present in the twenty-two rats which died spontaneously during the study. Their death was not due to PVM infection. Congestion and hemorrhage of the lung were the most common findings in these animals. The cause of death in most of these cases is not known, but exposure to RP/BR may have been a contributing factor.

Three animals which died spontaneously after day 51 had erosions of the laryngeal mucosa with deposits of fibrin on the surface. This fibrin probably partially obstructed the larynx and may have contributed to the death of these three animals. They were all in Group C3 and the lesion is considered related to RP/BR exposure."

5.4.1.2. Clinical Observations, Body Weights and Food Consumption

Prior to death several rats exposed to the high concentration exhibited labored breathing, wheezing, hunched posture and lethargy. The most frequent clinical observation not associated with lethality was discharge from the eye(s) with or without crust formation and/or swollen eye lids. Other infrequent findings included diarrhea and discharge from the nose or mouth. All these signs were considered to be not treatment-related based on the frequency of occurrence and their distribution in all test groups during the exposure and during the recovery period.

Statistically significant ($p < 0.001$) decreases in body weights were observed from Week 1 through Week 13 (Table 9). This effect was restricted to the 0.75- and 1.20-mg/L exposure groups from Weeks 1 to 13. For rats from the recovery groups, statistically significant ($p < 0.02$) results were seen during Weeks 14 and 15 when the animals that had been exposed to 0.75 mg/L continued to have decreased body weights. No significant differences were found during Weeks 16 to 21 (Table 9). Additional post hoc comparisons revealed similar results when these data were expressed as body weight gains. In this case Weeks 1 to 12 were decreased for the 0.75- and 1.20-mg/L-exposure groups and Week 14 remained decreased for the 0.75-mg/L-exposed animals only (Table 10).

A significant ($p < 0.001$) effect of treatment on food consumption was seen at Week 4. Post hoc comparisons revealed a significant decrease at Week 4 in the 0.75- and 1.20-mg/L-exposure groups. All other time points were non-significant (Table 11).

Table 9

FIRST STUDY

EFFECTS OF 13-WEEK SUBCHRONIC EXPOSURES^a TO RP/BR AEROSOLS ON
WEEKLY BODY WEIGHTS (G) OF MALE SPRAGUE-DAWLEY RATS
TESTED THROUGHOUT THE EXPOSURES AND THROUGH AN 8-WEEK RECOVERY PERIOD
(MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS))

TEST WEEK	0.0 mg/L	0.3 mg/L	0.75 mg/L	1.2 mg/L
PRETEST	198 ± 25 (164)	198 ± 26 (84)	196 ± 27 (164)	194 ± 27 (164)
1	234 ± 23 (164)	234 ± 22 (84)	224 ± 22 (164)*	210 ± 20 (162)*
2	265 ± 22 (164)	264 ± 22 (84)	253 ± 22 (163)*	242 ± 26 (150)*
3	292 ± 23 (164)	291 ± 23 (84)	279 ± 23 (161)*	272 ± 24 (149)*
4	315 ± 24 (163)	314 ± 24 (84)	300 ± 23 (160)*	294 ± 25 (149)*
5	331 ± 25 (164)	331 ± 25 (84)	317 ± 24 (161)*	309 ± 26 (149)*
6	344 ± 27 (164)	343 ± 25 (84)	329 ± 26 (161)*	324 ± 28 (149)*
7	357 ± 29 (164)	356 ± 26 (84)	340 ± 27 (161)*	335 ± 29 (149)*
8	368 ± 30 (164)	367 ± 28 (84)	351 ± 28 (161)*	346 ± 30 (148)*
9	377 ± 30 (164)	379 ± 28 (84)	361 ± 29 (161)*	357 ± 31 (147)*
10	385 ± 31 (164)	391 ± 29 (84)	369 ± 30 (161)*	365 ± 33 (147)*
11	392 ± 31 (163)	393 ± 29 (84)	377 ± 30 (161)*	373 ± 34 (147)*
12	398 ± 32 (163)	399 ± 29 (84)	384 ± 30 (161)*	380 ± 34 (145)*
13	403 ± 32 (161)	404 ± 29 (84)	393 ± 30 (161)*	390 ± 34 (144)*
14	404 ± 32 (80)	---- ± 0 (0)	389 ± 27 (78)*	395 ± 36 (73)
15	410 ± 34 (80)	---- ± 0 (0)	399 ± 27 (78)*	403 ± 38 (73)
16	418 ± 34 (80)	---- ± 0 (0)	407 ± 29 (77)	412 ± 38 (73)
17	420 ± 33 (80)	---- ± 0 (0)	411 ± 27 (77)	415 ± 39 (73)
18	422 ± 34 (80)	---- ± 0 (0)	414 ± 29 (77)	417 ± 39 (73)
19	426 ± 34 (79)	---- ± 0 (0)	423 ± 29 (77)	425 ± 39 (73)
20	430 ± 33 (79)	---- ± 0 (0)	424 ± 28 (77)	428 ± 40 (73)
21	435 ± 35 (79)	---- ± 0 (0)	429 ± 30 (77)	434 ± 40 (73)

*SIGNIFICANTLY DIFFERENT (P<0.05) FROM CORRESPONDING CONTROL GROUP
^a2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK

Table 10

FIRST STUDY

EFFECTS OF 13-WEEK SUBCHRONIC EXPOSURES^a TO RP/BR AEROSOLS ON
WEEKLY BODY WEIGHT GAINS (G) OF MALE SPRAGUE-DAWLEY RATS
TESTED THROUGHOUT THE EXPOSURES AND THROUGH AN 8-WEEK RECOVERY PERIOD
(MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS))

TEST WEEK	0.0 mg/L	0.3 mg/L	0.75 mg/L	1.2 mg/L
1	36 ± 9 (164)	36 ± 9 (84)	29 ± 10 (164)*	17 ± 15 (162)*
2	66 ± 13 (164)	66 ± 13 (84)	58 ± 14 (163)*	50 ± 18 (150)*
3	94 ± 17 (164)	93 ± 17 (84)	84 ± 17 (161)*	79 ± 19 (149)*
4	117 ± 23 (163)	116 ± 22 (84)	105 ± 23 (160)*	102 ± 24 (149)*
5	133 ± 25 (164)	133 ± 24 (84)	122 ± 23 (161)*	116 ± 25 (149)*
6	146 ± 28 (164)	146 ± 25 (84)	133 ± 27 (161)*	131 ± 27 (149)*
7	159 ± 29 (164)	158 ± 26 (84)	145 ± 27 (161)*	143 ± 29 (149)*
8	169 ± 31 (164)	170 ± 29 (84)	156 ± 28 (161)*	154 ± 30 (148)*
9	179 ± 32 (164)	181 ± 31 (84)	165 ± 30 (161)*	165 ± 31 (147)*
10	187 ± 33 (164)	193 ± 34 (84)	174 ± 31 (161)*	173 ± 32 (147)*
11	194 ± 33 (163)	196 ± 32 (84)	182 ± 32 (161)*	181 ± 33 (147)*
12	200 ± 34 (163)	201 ± 32 (84)	188 ± 32 (161)*	188 ± 34 (145)*
13	206 ± 35 (161)	207 ± 34 (84)	197 ± 33 (161)	198 ± 33 (144)
14	206 ± 33 (80)	----- ± 0 (0)	193 ± 29 (78)*	203 ± 37 (73)
15	212 ± 36 (80)	----- ± 0 (0)	203 ± 29 (78)	212 ± 34 (73)
16	221 ± 36 (80)	----- ± 0 (0)	211 ± 34 (77)	220 ± 37 (73)
17	222 ± 37 (80)	----- ± 0 (0)	215 ± 33 (77)	223 ± 38 (73)
18	225 ± 36 (80)	----- ± 0 (0)	218 ± 32 (77)	226 ± 37 (73)
19	228 ± 34 (79)	----- ± 0 (0)	224 ± 32 (77)	233 ± 37 (73)
20	232 ± 36 (79)	----- ± 0 (0)	228 ± 33 (77)	237 ± 39 (73)
21	237 ± 35 (79)	----- ± 0 (0)	233 ± 33 (77)	243 ± 37 (73)

*SIGNIFICANTLY DIFFERENT ($P < 0.05$) FROM CORRESPONDING CONTROL GROUP
^a2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK

Table 11

FIRST STUDY

EFFECTS OF 13-WEEK SUBCHRONIC EXPOSURES^a TO RP/BR AEROSOLS ON
FOOD CONSUMPTION (G/DAY) OF MALE SPRAGUE-DAWLEY RATS MEASURED
AT 4-WEEK INTERVALS THROUGHOUT THE EXPOSURES AND THE 8-WEEK RECOVERY PERIOD
(MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS))

TEST WEEK	0.0		0.3		0.75		1.2	
	mg/L		mg/L		mg/L		mg/L	
4	25 ±	4 (48)	24 ±	3 (24)	22 ±	3 (48)*	22 ±	3 (40)*
8	22 ±	3 (48)	24 ±	3 (24)	23 ±	4 (48)	23 ±	2 (40)
12	25 ±	4 (48)	25 ±	3 (24)	24 ±	3 (48)	25 ±	3 (40)
16	24 ±	2 (24)	---- ±	0 (0)	25 ±	3 (24)	24 ±	3 (21)
20	24 ±	3 (24)	---- ±	0 (0)	24 ±	2 (24)	24 ±	3 (21)

*SIGNIFICANTLY DIFFERENT ($P < 0.05$) FROM CORRESPONDING CONTROL GROUP
a=2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK

5.4.1.3. Pathology

5.4.1.3.1. Interim necropsies

The primary exposure-related change seen histologically in the lungs of animals examined at the interim necropsies at Weeks 2 and 4 during this subchronic study was diagnosed as "terminal bronchiolar fibrosis". The lesion consisted of a minimal thickening of the alveolar walls where the terminal bronchiole joins the alveolar sacs. The thickening consisted of a heterogeneous eosinophilic material which contained small numbers of cells. Masson's trichrome stain demonstrated small amounts of collagen in these areas which correlated with the degree of fibrosis seen with H&E stain. All interim-terminated animals exposed to 0.75 or 1.20 mg/L developed terminal bronchiolar fibrosis by four weeks of exposure (Table 12). The change was usually minimal in the 0.75- and moderate in the 1.20-mg/L-treatment groups, suggesting a dose-response. After two weeks of exposure all animals exposed to 1.20 mg/L had minimal to mild fibrosis but only 3 of 6 rats at 0.75 mg/L level had detectable thickening. In none of the animals that were exposed to 0.75 mg/L for two weeks could an increase in collagen be demonstrated with the trichrome stain.

Interstitial inflammation of the pulmonary parenchyma was present in more exposed animals that received 1.20 mg/L than in control or 0.75-mg/L-exposed animals. Other changes in the necropsied animals occurred in approximately equal frequency between exposed and control animals.

5.4.1.3.2. Terminal and recovery necropsies

There were no exposure-related changes found in any of the tissues which were examined outside of the respiratory tract. Lesions in general were infrequent and occurred in approximately the same incidence in animals exposed to the high concentration as in control animals.

The primary exposure-related change seen histologically after termination of this first subchronic study was in the lung and was diagnosed as "terminal bronchiolar fibrosis". The lesion consisted of thickening of the alveolar walls and of the most distal portions of the terminal bronchioles at the point where the terminal bronchiole ends and joins the alveolar sacs. The thickening consisted of a heterogeneous eosinophilic material containing small numbers of cells. Staining with Masson's trichrome stain showed strong evidence of collagen deposition. The incidence of this lesion by severity, summarized for interim, terminal and recovery necropsies shown in Table 12 demonstrates that inhalation of RP/BR for 2 weeks produced minimal bronchiolar fibrosis in 50% of rats exposed to 0.75 mg/L and minimal to mild

Table 12

INCIDENCE^a OF TERMINAL BRONCHIOLAR FIBROSIS IN THE FIRST
SUBCHRONIC STUDY

Severity Of Lesions	Exposure Periods and RP/BR Concentrations (mg/L)							
	2 Weeks ^b		4 Weeks ^b		13 Weeks ^c			Recovery ^d
	(0.75)	(1.20)	(0.75)	(1.20)	(0.30)	(0.75)	(1.20)	(0.75) (1.20)
Minimal	3/6	5/6	5/6		4/12	3/12	1/12	5/12
Mild		1/6	1/6	1/5		9/12	1/12	7/12 3/12
Moderate				4/5			6/12	7/12
Severe							4/12	2/12

^aNumber of animals having lesion/total number examined in the exposure group

^bInterim necropsy

^cTerminal necropsy after completion of last exposure

^dTerminal necropsy after 8 weeks of recovery following last exposure

fibrosis in all of the 1.2-mg/L-exposed rats. All rats had fibrosis after 4 weeks of exposure to 0.75 or 1.2 mg/L of RP/BR. After completion of the 13 week study, 100% of the rats exposed to 0.75 mg/L RP/BR or higher and approximately 30% of rats exposed to 0.30 mg/L had terminal bronchiolar fibrosis. Severity and frequency of the lesion increased with the higher concentrations. The incidence of fibrosis in the recovery animals was only slightly less than that of the animals killed immediately after the exposure and does not suggest resolution of the lesion with time.

5.4.2. Second Study

5.4.2.1. Spontaneous Deaths

No animals died during the exposure phase of the second study (Weeks 1 to 13) but two died during the 8-week recovery period. One rat from the 0.18-mg/L-exposure group developed a mass on the hip which later spread to the leg. The mass was first apparent at Test Week 10 and the rat died at Test Week 15. This animal had a histocytic sarcoma, which was probably contributory to its death. In addition, one rat from the 0.30-mg/L-exposure group died during Test Week 18 following a marked body weight loss. This animal had chronic hemorrhage and congestion of the lungs. These mortalities and the development of a mass were considered as spurious events and not a consequence of exposures to RP/BR.

5.4.2.2. Clinical Observations and Body Weights

The most frequently observed clinical sign was red/crusted eye(s). Other clinical signs sporadically seen included watery eye(s), lesions on head, neck, tail and extremities. None of these signs were judged to be exposure-related based on their distribution and frequency of occurrence.

Statistically significant increases in body weights were observed for the 0.05-mg/L-exposure group relative to controls during Weeks 4 through 12 (Table 13) (group by time interaction $p < 0.0001$). In terms of body weight gains, rats from the 0.18- and 0.30-mg/L-exposure groups showed significant decreases on Test Week 1, whereas the 0.05-mg/L-exposed animals showed significant increases on Test Weeks 3 to 12, 15 and 20 and rats from the 0.18-mg/L-exposure group demonstrated significant increases on Test Weeks 5 to 11, 20 and 21 (Table 14).

Table 13

SECOND STUDY

EFFECTS OF 13-WEEK SUBCHRONIC EXPOSURES^a TO RP/BR AEROSOLS ON
WEEKLY BODY WEIGHTS (G) OF MALE SPRAGUE-DAWLEY RATS
TESTED THROUGHOUT THE EXPOSURES AND THROUGH AN 8-WEEK RECOVERY PERIOD
[MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS)]

TEST WEEK	0.0 mg/L	0.05 mg/L	0.18 mg/L	0.30 mg/L
PRETEST	233 ± 25 (124)	231 ± 24 (124)	231 ± 24 (124)	231 ± 24 (124)
1	265 ± 19 (124)	262 ± 22 (124)	259 ± 22 (124)	259 ± 20 (124)
2	289 ± 20 (124)	291 ± 21 (124)	286 ± 24 (124)	284 ± 22 (124)
3	312 ± 24 (124)	317 ± 23 (124)	314 ± 25 (124)	309 ± 23 (124)
4	331 ± 25 (124)	339 ± 21 (124)*	335 ± 25 (124)	331 ± 24 (124)
5	346 ± 26 (124)	355 ± 23 (124)*	353 ± 26 (124)	347 ± 24 (124)
6	362 ± 28 (124)	370 ± 24 (124)*	368 ± 28 (124)	363 ± 26 (124)
7	373 ± 29 (124)	383 ± 24 (124)*	381 ± 28 (124)	374 ± 26 (124)
8	383 ± 30 (124)	393 ± 25 (124)*	391 ± 28 (124)	384 ± 27 (124)
9	393 ± 30 (124)	403 ± 26 (124)*	400 ± 29 (124)	394 ± 29 (124)
10	402 ± 30 (124)	411 ± 25 (124)*	409 ± 29 (124)	403 ± 27 (124)
11	409 ± 30 (124)	419 ± 25 (124)*	416 ± 30 (124)	411 ± 28 (124)
12	416 ± 31 (124)	425 ± 26 (124)*	422 ± 30 (124)	419 ± 29 (114)
13	422 ± 31 (124)	428 ± 26 (121)	427 ± 30 (124)	425 ± 29 (124)
14	429 ± 33 (62)	438 ± 26 (62)	433 ± 33 (62)	430 ± 29 (62)
15	433 ± 34 (62)	446 ± 26 (62)	438 ± 28 (61)	436 ± 30 (52)
16	434 ± 35 (62)	446 ± 25 (62)	440 ± 29 (61)	435 ± 30 (52)
17	437 ± 34 (62)	445 ± 25 (62)	441 ± 28 (61)	442 ± 31 (62)
18	433 ± 36 (62)	438 ± 24 (62)	435 ± 31 (61)	434 ± 32 (61)
19	430 ± 31 (62)	435 ± 27 (62)	432 ± 28 (61)	431 ± 28 (61)
20	438 ± 35 (62)	448 ± 27 (62)	447 ± 30 (61)	445 ± 29 (61)
21	450 ± 36 (62)	460 ± 25 (62)	459 ± 30 (61)	457 ± 30 (61)

*SIGNIFICANTLY DIFFERENT (P<0.05) FROM CORRESPONDING CONTROL GROUP
a=2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK

Table 14

SECOND STUDY

EFFECTS OF 13-WEEK SUBCHRONIC EXPOSURES^a TO RP/BR AEROSOLS ON
WEEKLY BODY WEIGHT GAINS (G) OF MALE SPRAGUE-DAWLEY RATS
TESTED THROUGHOUT THE EXPOSURES AND THROUGH AN 8-WEEK RECOVERY PERIOD
[MEAN AND STANDARD DEVIATION (NUMBER OF ANIMALS)]

TEST WEEK	0.0 mg/L	0.05 mg/L	0.18 mg/L	0.30 mg/L
1	32 ± 11 (124)	31 ± 10 (124)	29 ± 10 (124)*	27 ± 8 (124)*
2	56 ± 17 (124)	60 ± 12 (124)	55 ± 13 (124)	53 ± 12 (124)
3	79 ± 21 (124)	86 ± 15 (124)*	83 ± 14 (124)	78 ± 17 (124)
4	98 ± 26 (124)	108 ± 19 (124)*	104 ± 18 (124)	99 ± 21 (124)
5	114 ± 27 (124)	124 ± 21 (124)*	122 ± 20 (124)*	116 ± 22 (124)
6	129 ± 28 (124)	139 ± 22 (124)*	137 ± 21 (124)*	131 ± 23 (124)
7	141 ± 31 (124)	151 ± 24 (124)*	150 ± 23 (124)*	143 ± 25 (124)
8	150 ± 31 (124)	162 ± 24 (124)*	160 ± 24 (124)*	153 ± 26 (124)
9	160 ± 33 (124)	172 ± 24 (124)*	169 ± 24 (124)*	163 ± 30 (124)
10	169 ± 32 (124)	180 ± 25 (124)*	178 ± 25 (124)*	171 ± 28 (124)
11	176 ± 34 (124)	188 ± 25 (124)*	186 ± 27 (124)*	180 ± 29 (124)
12	183 ± 33 (124)	194 ± 26 (124)*	191 ± 27 (124)	189 ± 28 (114)
13	189 ± 34 (124)	198 ± 27 (121)	196 ± 27 (124)	194 ± 29 (124)
14	196 ± 36 (62)	206 ± 22 (62)	203 ± 25 (62)	200 ± 28 (62)
15	200 ± 39 (62)	214 ± 26 (62)*	208 ± 27 (61)	209 ± 32 (52)
16	201 ± 38 (62)	214 ± 27 (62)	211 ± 29 (61)	200 ± 30 (52)
17	204 ± 38 (62)	214 ± 27 (62)	212 ± 29 (61)	211 ± 33 (62)
18	199 ± 42 (62)	207 ± 32 (62)	206 ± 34 (61)	204 ± 37 (61)
19	197 ± 33 (62)	203 ± 24 (62)	203 ± 26 (61)	201 ± 27 (61)
20	205 ± 37 (62)	217 ± 23 (62)*	218 ± 24 (61)*	214 ± 24 (61)
21	216 ± 38 (62)	228 ± 26 (62)	230 ± 28 (61)*	226 ± 28 (61)

*SIGNIFICANTLY DIFFERENT (P<0.05) FROM CORRESPONDING CONTROL GROUP
a=2.25 HR/DAY ON 4 CONSECUTIVE DAYS/WEEK

5.4.2.3. Pathology

5.4.2.3.1. Post-exposure and post-recovery necropsies

The primary treatment-related change seen histologically in this second (lower concentration-range) subchronic study was still terminal bronchiolar fibrosis in the lung in rats from the 0.30- and 0.18-mg/L-treatment groups. The lesion consisted of minimal thickening of the alveolar walls and of the most distal portions of the terminal bronchioles at the point where the terminal bronchiole ends and joins the alveolar sacs. The thickening consisted of a heterogeneous eosinophilic material containing small numbers of cells. This material stained positively for collagen with Masson's trichrome stain. The incidence of this lesion summarized in Table 15 demonstrates that minimal terminal bronchiolar fibrosis was found in less than 50% and 25% of the rats exposed to 0.30 and 0.18 mg/L of RP/BR, respectively, with a total absence of the lesions in the 0.05-mg/L-exposure group. Although the incidence of fibrosis was somewhat decreased after the recovery period, it nevertheless did not disappear.

Other changes in the lung were usually minimal or absent altogether. Some animals had small numbers of alveolar macrophages but they were usually not associated with the terminal bronchiolar fibrosis. Minimal to mild lymphocytic hyperplasia occurred in many of the animals, both treated and controls. Other changes were relatively infrequent.

Table 15

INCIDENCE^a OF TERMINAL BRONCHIOLAR FIBROSIS IN THE SECOND SUBCHRONIC STUDY

Severity Of Lesions	Exposure Periods and RP/BR Concentrations (mg/L)					
	13-Week Exposure ^b			8-Week Recovery ^c		
	(0.05)	(0.18)	(0.30)	(0.05)	(0.18)	(0.30)
Minimal	0/20	4/20	9/20	0/20	3/20	4/20
Mild	-	-	-	-	-	-
Moderate	-	-	-	-	-	-
Severe	-	-	-	-	-	-

^aNumber of animals having lesion/total number examined in the exposure group

^bTerminal necropsy after completion of last exposure

^cTerminal necropsy after 8 weeks of recovery following last exposure

6. SUMMARY DISCUSSION

In its concern for personnel health and safety, the U.S. Army Medical Research and Development Command initiated a research program to provide a comprehensive definition of the biological effects of red phosphorus/butyl rubber smoke on mammalian systems under conditions which approximate the potential troop exposure. The studies used laboratory rats placed into controlled test atmospheres in whole-body exposure chambers and were conducted in four phases.

In Phase I inhalation exposure facilities with automatically controlled conditioned air supply and exhaust systems were built and specially designed RP/BR combustion generators provided by the Government were installed. Aerosol sampling methods for monitoring of mass concentration, particle size and percentage phosphorous acids were established and used to characterize the aerosol exposure system for spatial and temporal homogeneity. Phase II consisted of range-finding acute and repeated-dose exploratory studies to determine lethality (LC_{50}) and influence of exposure duration on morbidity; whereas in Phase III the combination of the effects of exposure concentration, duration and frequency on a series of biological endpoints were examined in depth after 4-week exposure and 2-week recovery periods, to define time-concentration relationships as well as threshold levels, healing, and adaptation in biological responses.

The objective of the Phase IV studies, the subject of the current final report, was to evaluate the biological effects and the reversibility of the observed effects in subchronic RP/BR aerosol exposures on various biological endpoints in male rats. The primary objective was to define the no-measurable effect level for the biological response parameters assessed to be the most sensitive indicators of dysfunction. The experimental design and the exposure conditions were developed based upon the results of the Phase III studies and included exposures for 2.25 hr/day on four consecutive days per week for a period of 13 weeks to 0.30 (C_1), 0.75 (C_2) and 1.20 (C_3) mg/L of RP/BR aerosol, or to filtered air (C_0). The experimental endpoints selected for evaluation of the effects after 13 weeks of exposure and for the C_2 , C_3 and C_0 treatment groups after an 8-week recovery period included mortality, clinical observations, body weights, food consumption, histopathology of the lungs and other major organs, examination of the pulmonary free cells collected from the lungs by lavage, measurement of *in vivo* pulmonary bactericidal activity and evaluation of neurobehavioral activity parameters. The biological and statistical evaluation of this study showed that the no-measurable effect level had not been reached. The results demonstrated that although some significant changes could be observed for almost all parameters under some of the conditions examined, the most striking and consistent effects found for all three RP/BR concentration levels were the histopathologically determined terminal bronchiolar fibrosis and the highly

significant ³⁵S-decrease measured in pulmonary bactericidal activity to inhaled ³⁵S-Klebsiella pneumoniae.

Therefore, these two experimental parameters were used as major biological endpoints in a second subchronic study conducted at aerosol concentrations lowered to 0.05, 0.18 and 0.30 mg/L, (thus repeating the lowest level from the previous study as the highest concentration), to further explore the fibrotic changes in the lungs and to examine the effects on pulmonary bactericidal activity after a 13-week exposure and an 8-week recovery period.

Throughout the two subchronic studies aerosol mass concentrations were within 3% of the target values, MMAD's ranged from 0.49 to 0.65 μ m with σ 's of 1.56 to 1.83, while phosphorous acid levels in the test atmosphere varied from 71 to 80%.

During the first study 10.8% of the rats exposed to 1.20 mg/L died spontaneously or were necropsied in a moribund state. In addition statistically significant decreases in body weights and body weight gains were observed from weeks 1 through 13 in the 0.75- and 1.20-mg/L-exposure groups. A significant decrease in food consumption was only seen after four weeks of exposure in these groups.

Although positive antibody titers to PVM were found in rats from the first subchronic study, no lesions characteristic of PVM infections were found in the lungs of the rats that died spontaneously or in those examined at terminal necropsy and neither the deaths nor the lesions found in the lungs were a consequence of PVM infection.

Neurobehavioral activity measurements conducted according to the experimental protocol did not show consistent or significant changes. Statistically significant changes in pulmonary lavage parameters found in the first study were sporadic and not considered biologically significant.

Significant decreases in pulmonary bactericidal activity to inhaled ³⁵S- K. pneumoniae at all three exposure concentrations (1.20, 0.75 and 0.30 mg/L) of the first study were completely absent after the recovery, whereas in the second study none of the RP/BR exposures (0.30, 0.18 and 0.05 mg/L), including the previously positive 0.30 mg/L, produced an effect. Thus the no-measurable effect level for pulmonary bactericidal activity was \leq 0.30 mg/L of RP/BR aerosol.

Histologically no treatment-related changes were found in any of the tissues examined outside of the respiratory tract. The primary treatment-related change seen after 13 weeks of exposure in both subchronic studies was terminal bronchiolar fibrosis in the lung. Strong evidence for fibrosis was the collagen deposition at the affected sites as shown by Masson's trichrome stain.

Inhalation of RP/BR in male rats for 2.25 hr/day for 4 days/week begins to produce fibrosis after two weeks in some of the rats exposed to 0.75 mg/L and in all of those exposed to 1.2 mg/L. All animals had fibrosis at both of these concentrations after four weeks. At the end of 13 weeks, in addition to 100% of the 0.75- and 1.20-mg/L-treatment groups, less than 50% of those exposed to 0.30 mg/L and less than 25% of those that inhaled 0.18 mg/L were also affected. Exposure to 0.05 mg/L did not produce fibrotic changes at all. Some decrease in incidence of the fibrotic lesions could be generally detected after the recovery periods, but the lesions did not disappear.

An overall review of the results of the two studies demonstrates that in the first study 10.8% of the rats exposed to 1.20 mg/L of RP/BR smoke died spontaneously or were necropsied in a moribund state. Most of these animals died during the first two weeks of the exposures, and had varying degrees of congestion and small amounts of hemorrhage in the lungs. Those RP/BR-exposed (0.75 and 1.20 mg/L) animals which died during the later parts of the study, had terminal bronchiolar fibrosis and erosions of the laryngeal mucosa with deposition of fibrin on the surface. The laryngeal changes were probably contributory to their death. The presence of congestion, hemorrhage, and interstitial inflammation in the lungs of 1.20-mg/L-exposed rats which died during this experiment strongly suggests that these effects were due to the RP/BR smoke. This concentration obviously produces morbidity, since, in addition to these morphologic changes, decreased body weight and food consumption were measured. At the terminal necropsy all animals had terminal bronchiolar fibrosis in the 0.75- and 1.20-mg/L-exposure groups and all three exposure groups had significantly reduced pulmonary bactericidal activity.

In the second study at the concentrations of 0.30, 0.18 and 0.05 mg/L RP/BR there were no exposure-related mortalities and neither pulmonary bactericidal activity nor body weights showed significant decreases. Terminal bronchiolar fibrosis was still found in the lungs of rats exposed to the medium and high doses but the lesions were minimal in severity and were totally absent in the 0.05-mg/L-exposure group.

Thus, comparison of the findings of the two studies demonstrates that there is a steep dose response curve for the test material upon repeated exposures: The responses at 1.20 and 0.75 mg/L include lethality in addition to terminal bronchiolar fibrosis and significantly reduced pulmonary bacterial activity, body weights and food consumption whereas at approximately a tenfold lower level (between 0.05 and 0.18 mg/L) there is no measurable effect.

In summary, the results of these Phase IV studies demonstrate that when male Sprague Dawley rats inhaled RP/BR combustion products for 2.25 hr/day on four days per week for 13 weeks, the

lowest effective dose was determined as 0.10 mg/L and the no-measurable effect level was identified as 0.05 mg/L through evaluation of fibrotic changes in the lung.

PUBLICATIONS AND PRESENTATIONS RESULTING FROM THIS CONTRACT

Publications

Studies with aerosols of red phosphorus/butyl rubber combustion products. Proceedings: Smoke/Obscurants Symposium VIII, Vol. I, 351-368, 1984. C. Aranyi, S. C. Vana and R. Gibbons. Effects of inhalation of red phosphorus/butyl rubber (RP/BR) combustion products in rats. Proceedings: Smoke and Obscurants Symposium IX, Vol. II, 643-654, 1985. C. Aranyi, S. Vana and R. D. Gibbons. Pulmonary responses after inhalation of red phosphorus/butyl rubber combustion products in rats. Proceedings: Smoke and Obscurants Symposium X, 2, 631-639, 1986. C. Aranyi.

Abstracts

Inhalation of a complex mixture of red phosphorus/butyl rubber combustion products. Poster presented at the 23rd Annual Meeting of the Society of Toxicology, March, 1984. Atlanta, GA. C. Aranyi, J. N. Bradof and S. C. Vana. Effects of short-term inhalation exposures to red phosphorus butyl rubber (RP/BR) combustion products in rats. Poster presented at the 24th Ann. Meeting of the Soc. of Toxicology, San Diego, CA. March 1985. C. Aranyi, J. N. Bradof, M. Furedi, R. L. Sherwood and S. C. Vana. Effects of inhalation of red phosphorus/butyl rubber (RP/BR) combustion products on the pulmonary cellular responses of rats. Poster presented at the Joint Conf. of the 17th Inter. Leukocyte Culture Conf. and the 22nd Nat. Meeting of the Reticuloendothelial Soc., Ithaca, NY, August 1985. C. Aranyi, J. N. Bradof and R. L. Sherwood. Effects of alveolar macrophages of rats inhaling red phosphorus butyl rubber (RP/BR) combustion products. Poster presented at the 25th Annual Meeting of the Soc. of Toxicology, New Orleans, LA, March 1986. C. Aranyi, J. N. Bradof and R. L. Sherwood.

Presentations and Submitted Publications

Effects of inhalation of red phosphorus/butyl rubber combustion products on alveolar macrophage responses in rats. Presented at the Symposium "Selected Nuisance Dusts: Are Short Term Tests Predictive?" Nat. Capital Area Chapter, SOT. November 1985. College Park, MD. C. Aranyi and R. I. Sherwood. Research and development on inhalation toxicologic evaluation of red phosphorus/butyl rubber combustion products. US Army Medical Bioengineering Research and development Laboratory, 1985 Extramural Review. Presentation. C. Aranyi. Pulmonary responses after inhalation of red phosphorus/butyl rubber combustion products in rats. Proceedings: Smoke and Obscurants Symposium X, 1986. Presentation. C. Aranyi.

Effects of inhalation of red phosphorus/butyl rubber combustion products on alveolar macrophage responses in rats. Submitted to J. Appl. Tox. C. Aranyi, S.C. Vana, J.N. Bradof, and R.L. Sherwood.

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APPENDIX A
MORPHOLOGICAL PATHOLOGY

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IIT PROJECT NUMBER L06139
PHASE IV STUDY NO. 80

THE EFFECTS OF SUBCHRONIC EXPOSURES TO
RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
ON VARIOUS BIOLOGICAL ENDPOINTS
IN MALE SPRAGUE-DAWLEY RATS

GROSS NECROPSY OBSERVATIONS

In accordance with experimental protocol, gross examinations of organs and tissues were performed on 141 (including 22 spontaneous deaths) male Sprague-Dawley rats in the toxicology group of Project L06139 Study Number 80. The rats were divided into 13 groups each containing male rats. The rats were exposed to various concentrations of RP/BR aerosol for 2.25 hours per day for four consecutive days for two, four or 13 week periods. Ten of the 13 groups of rats were sacrificed on the day of their last exposure while the rats in the remaining three groups (the recovery groups) were sacrificed 8 weeks following their last exposure. The groups, treatment, number of rats per group, and corresponding exposure concentration levels are outlined below.

Treatment	Weeks Exposed	Number of Rats	Exposure Concentration Levels (mg/l)
Filtered Air	13	13 ^a	0.00
RP/BR Aerosol	13	12	0.30
RP/BR Aerosol	13	12	0.75
RP/BR Aerosol	13	23 ^a	1.20
Filtered Air	13 ^b	13 ^a	0.00
RP/BR Aerosol	13 ^b	13 ^a	0.75
RP/BR Aerosol	13 ^b	19 ^a	1.20
Filtered Air	2	6	0.00
RP/BR Aerosol	2	6	0.75
RP/BR Aerosol	2	6	1.20
Filtered Air	4	6	0.00
RP/BR Aerosol	4	6	0.75
RP/BR Aerosol	4	6 ^a	1.20

^a Including spontaneous deaths.

^b Exposures followed by 8 weeks of recovery.

MATERIALS AND METHODS

The rats were anesthetized with Nembutal, exsanguinated by way of the abdominal aorta and necropsied. The organs were examined and fixed in 10% neutral buffered formalin for a period of no less than 48 hours before further processing. The lungs were fixed by intratracheal perfusion with formalin.

The following tissues were collected at necropsy. Tissues marked with an asterisk (*) in the list below were processed by the Histology Laboratory embedded in paraffin and resulting blocks were sent to EPL for further processing and microscopic examination.

Skin/Mammary Gland	Ileum	*Liver
Tongue	Jejunum	*Kidneys
*Larynx	Mandibular Lymph Nodes	*Adrenal Glands
Parathyroid/Thyroid	*Eyes	Spleen
*Trachea	Brain	Pancreas
*Esophagus	Spinal Cord (Cervical)	Cecum
*Heart	Pituitary Gland	Colon
Thymus	Ears (Tag)	Mesenteric Lymph
*Lungs	*Nasal Turbinates	Nodes
*Urinary Bladder	*Respiratory Lymph Nodes	Skeletal Muscle
*Stomach	Sternum	Sciatic Nerve
*Duodenum	*Testes	Salivary Glands
Femur/Bone Marrow		Mandibular

A summary of gross observations is presented by groups in the Necropsy Observation Tables (Tables I - V).

GROSS PATHOLOGY RESULTS

Treatment-related lesions were observed in rats that were exposed for 2 and 4 weeks to 1.2 mg/l RP/BR and that died spontaneously during the study. Treatment-related lesions seen in animals after 2 or 4 weeks of exposure included red areas, dark red foci or gray areas in the lung, mottled red thymus, dark red mandibular lymph node and dark red focus in the mesenteric lymph node. No substantial differences were seen in the incidence of gross findings between control and treated animals sacrificed

after 13 weeks of exposure or after the recovery period. The dilated pelvis and flaccid kidneys seen in two treated rats are spontaneous changes which are occasionally seen in rats of this strain and are not considered treatment-related. Treatment-related changes which occurred in animals which died spontaneously included mottled dark red lungs, dark red fluid in the abdominal and thoracic cavities, intestine and brain, and dark red livers and spleens.

SUMMARY AND CONCLUSIONS

Treatment-related changes were observed primarily in the lungs of rats exposed for 2 and 4 weeks and those that died during the study. Changes in color and abnormal accumulations of dark red fluid in body cavities were additional lesions seen in occasional animals which may also be treatment-related.

W. O. Iverson, D.V.M.
W.O. Iverson, D.V.M.
Diplomate, ACVP

March 8, 1945

GROSS NECROPSY OBSERVATIONS TABLES

TABLE 1

6139 GROSS NECROPSY OBSERVATIONS
 Study Number 80 Male Rats Sacrificed After Two Weeks of Study
 Exposure Frequency: F₂

ORGAN Lesion	Exposure Concentration (mg/l)		
	0.0	0.75	1.20
NUMBER OF RATS EXAMINED			
NO GROSS LESIONS	6	6	6
	6	6	2
LUNGS			
Red area			
Dark red focus			1/6
Gray red area			1/6
			1/6
THYMUS			
Mottled red			1/6
MANDIBULAR LYMPH NODE			
Dark red			1/6
MESENTERIC LYMPH NODE			
Dark red focus			1/6

Duration of exposure 2.25 hours.

GROSS NECROPSY OBSERVATIONS

Exposure Frequency: F₂

Lesion

[illegible]

82

L6139
Study Number 80
Exposure Frequency: F₂

Duration of exposure 2.25 hours.

TABLE IV

GROSS NECROPSY OBSERVATIONS
Male Rats Sacrificed After 8 Weeks of Recovery

L6139
Study Number 80

Exposure Frequency: F₂

ORGAN Lesion	Exposure Concentration (µg/l)		
	0.0	0.75	1.20
NUMBER OF RATS EXAMINED	12	12	12
NO GROSS LESIONS	11	9	9
LUNGS			
Red foci		1/12	
KIDNEYS			
Flacid			2/12
Dilated pelvis			1/12
URINARY BLADDER			
Contained calculi	1/12		
TESTES			
Small		1/12	
PREPUTIAL GLAND			
Abscess			1/12
ABDOMINAL FAT			
Fat necrotic		1/12	

TABLE V

GROSS NECROPSY OBSERVATIONS

Male Rats that Died Spontaneously During Study
or Were Sacrificed Moribund

L6139

Study Number 80

Exposure Frequency: F₂

ORGAN

Lesion

NUMBER OF RATS EXAMINED	Exposure Concentration (µg/l)		
	0.0	0.75	1.20
LUNGS			
Dark red			1/19
Dark red areas		1/1	1/19
Mottled dark red	1/2		18/19
THORACIC CAVITY			
Contained dark red fluid		1/1	11/19
HEART			
Firm	1/2	1/1	1/19
Enlarged	1/2		
THYMUS			
Mottled dark red	1/2		
BRAIN			
Dark red fluid			2/19

Duration of exposure 2.25 hours.

TABLE V (cont.)

GROSS NECROPSY OBSERVATIONS
Male Rats that Died Spontaneously During Study
or Were Sacrificed Moribund

L6139

Study Number 80

Exposure Frequency: F₂

ORGAN Lesion	Exposure Concentration (µg/l)		
	0.0	0.75	1.20
MANDIBULAR LYMPH NODES			
Dark red	1/2		
ABDOMINAL CAVITY			
Contained dark red fluid			3/19
LIVER			
Dark red	2/2		18/19
KIDNEYS			
Mottled dark red	1/2		
Soft		1/1	
Raised four areas			1/19
SPLEEN			
Dark red	1/2		4/19

Duration of exposure 2.25 hours.

TABLE V (cont.)

GROSS NECROPSY OBSERVATIONS
Male Rats that Died Spontaneously During Study
or Were Sacrificed Moribund

L6139
Study Number 80

Exposure Frequency: F₂

ORGAN	Lesion	Exposure Concentration (µg/l)		
		0.0	0.75	1.20
ADRENALS				
	Red	1/2		
	Enlarged	1/2		
MESENTERIC LYMPH NODES				
	Dark red	1/2		
INTESTINES				
	Contained dark red fluid			2/19
	Empty (except cecum)			2/19
URINARY BLADDER				
	Contained calculi		1/1	1/19
	Dark red fluid			1/19
STOMACH				
	Gas-filled, distended			12/19
	Dark red			1/19

Duration of exposure 2.25 hours.

TABLE V (cont.)

GROSS NECROPSY OBSERVATIONS
Male Rats that Died Spontaneously During Study
or Were Sacrificed Moribund

L6139
Study Number 80
Exposure Frequency: F₂

ORGAN Lesion	Exposure Concentration (µg/l)		
	0.0	0.75	1.20
DUODENUM			
Gas-filled, empty			1/19
JEJUNUM			
Gas-filled, empty			3/19
ILEUM			
Gas-filled, empty			3/19
Yellow fluid			1/19
CECUM			
Gas-filled, bloated	1/2	1/1	
Dark red			1/19
Contained dark red fluid			1/19
MANDIBULAR SALIVARY GLAND			
Dark red	1/2		

Duration of exposure 2.25 hours.

IITRI PROJECT NUMBER L06139
PHASE IV STUDY NO. 80
THE EFFECTS OF SUBCHRONIC EXPOSURES TO
RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
ON VARIOUS BIOLOGICAL ENDPOINTS
IN MALE SPRAGUE-DAWLEY RATS

PATHOLOGY REPORT

INTERIM SACRIFICES AND EARLY MORTALITIES

Submitted To:

IIT Research Institute
Chicago, IL 60616

November 29, 1984

QUALITY ASSURANCE
REPORT CERTIFICATION

Client Name: IIT Research Institute

Client Study Number: L06139, Phase IV, Study No. 80

Study Director: Dr. W.O. Iverson Pathologist: Dr. W.O. Iverson

Study Title: The Effects of Subchronic Exposure to Red Phosphorus/
Butyl Rubber (RP/BR) Combustion Products

Test Article: Combustion Product of Red Phosphorus/Butyl Rubber

Species: Sprague-Dawley Rat

All parts of the pathology phase of this study, including the final report, were reviewed by Experimental Pathology Laboratories Quality Assurance Unit on November 29, 1984. All findings were reported to the Study Director and Management.


Betty L. Plankenhorn

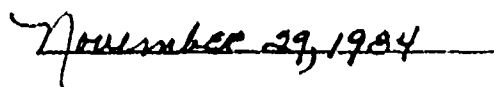

November 29, 1984

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PATHOLOGY SUMMARY

IIT PROJECT NUMBER L06139
 PHASE IV STUDY NO. 80
 THE EFFECTS OF SUBCHRONIC EXPOSURES TO
 RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
 ON VARIOUS BIOLOGICAL ENDPOINTS
 IN MALE SPRAGUE-DAWLEY RATS

PATHOLOGY SUMMARY

INTERIM SACRIFICES AND MORTALITIES

Microscopic examinations were performed on selected tissues from male Sprague-Dawley rats. The purpose of this study was to evaluate the effects of exposure concentration and recovery time of the repeated exposure of rats to combustion products of Red Phosphorus/Butyl Rubber (RP/BR) on various biologic endpoints. This report contains the histopathologic findings for the interim sacrifices and mortalities. The experimental design for this study was as follows:

T.G. NO.	EXPOSURE CODE	RECOV.	CONCENTRATION mg/L	ANIMAL NUMBERS*	DISPOSITION
41	C0	-	0.00	529-540	Final Sac
42	C1	-	0.30	541-552	Final Sac
43	C2	-	0.75	553-564	Final Sac
44	C3	-	1.20	565-576	Final Sac
45	C0	-	0.00	577-588	Final Sac
46	C1	-	0.30	589-600	Final Sac
47	C2	-	0.75	601-612	Final Sac
48	C3	-	1.20	613-624	Final Sac
49	C0	+	0.00	625-636	Recovery Sac
50	C2	+	0.75	637-648	Recovery Sac
51	C3	+	1.20	649-660	Recovery Sac
52	C0	+	0.00	661-672	Recovery Sac
53	C2	+	0.75	673-684	Recovery Sac
54	C3	+	1.20	685-696	Recovery Sac
67	C0	-	0.00	817-822	2-Week Interim Sac
68	C2	-	0.75	823-828	2-Week Interim Sac
69	C3	-	1.20	829-834	2-Week Interim Sac
70	C0	-	0.00	835-840	4-Week Interim Sac
71	C2	-	0.75	841-846	4-Week Interim Sac
72	C3	-	1.20	847-852	4-Week Interim Sac

T.G. NO. = Treatment group number

* Six selected rats from Treatment Groups Numbers 41-54 will be used for the necropsy and histopathology.

All animals were exposed for 2.25 hours per day for four consecutive days for two, four, or thirteen weeks. Recovery animals were then untreated for an additional eight weeks. All rats were necropsied and gross and histologic evaluations of the respiratory tract were conducted. According to protocol the lung from all animals was trimmed and processed to paraffin blocks. The paraffin blocks were then shipped to Experimental Pathology Laboratories, Inc. where hematoxylin and eosin stained slides were prepared and examined. Additional sections of all lungs from animals sacrificed at two and four weeks were also prepared and stained with Masson's trichrome stain to demonstrate collagen.

RESULTS

The microscopic changes found in all lung lobes examined are listed in the Histopathology Incidence Table. The primary treatment-related change seen histologically in the sections of lung examined from the interim sacrifice animals was diagnosed as "terminal bronchiolar fibrosis". The lesion consisted of a minimal thickening of the alveolar walls where the terminal bronchiole joins the alveolar sacs. The thickening consisted of a heterogeneous eosinophilic material which contained small numbers of cells. Masson's Trichrome stain demonstrated small amounts of collagen in these areas which correlated with the degree of fibrosis seen with H&E stain. Most animals exposed to 0.75 or 1.2 mg/l developed terminal bronchiolar fibrosis by four weeks of exposure. The change was usually minimal in Group 71 and moderate in Group 72, demonstrating a dose-response. After two weeks of exposure all animals exposed to 1.2 mg/l had minimal to mild fibrosis but only 3 of 6 rats at 0.75 mg/l had detectable thickening. In all of these Group 68 animals, an increase in collagen was not demonstrable with the trichrome stain.

Interstitial inflammation of the pulmonary parenchyma was present in more treated animals that received 1.2 mg/l than in C0 or C2 animals. Other changes in the sacrificed animals occurred in approximately equal frequency between treated and control animals.

A total of sixteen animals died spontaneously or were sacrificed in a moribund condition prior to their scheduled termination. Most animals had varying degrees of congestion and small amounts of hemorrhage in the lung tissue. Other lesions seen were similar to those found in the interim sacrificed animals. No obvious cause of death was apparent from examination of the lungs from these animals.

CONCLUSION

The results of these microscopic examinations indicate that the administration of RP/BR to male rats for 2.25 hr/day for four consecutive days begins to produce terminal bronchiolar fibrosis after two weeks of exposure at .75 mg/l. All animals exposed to 1.2 mg/l for two weeks had minimal fibrosis and most animals had fibrosis at both of these concentrations after four weeks. Interstitial inflammation was a dose-related lesion which occurred in some animals in the high dose group. A number of animals died or were sacrificed in a moribund condition during the first few weeks of the study. The cause of death could not be determined from examination of the lung tissue.

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November 29, 1984

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

Treatment Group
Exposure

PROJECT L06139
PHASE IV, STUDY NO. 80
MALE RATS

TWO WEEK SACRIFICE

NUMBER
ANIMAL

67 68 69
C0 C2 C3

	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834
LUNG																		
Hemorrhage																		
Atelectasis	2							2					2	2	1			
Alveolar Macrophages	1								1		1			2	1	1	1	
Eosinophilic Infiltrate												1	1				2	
Terminal Bronchiolar Fibrosis								1	1	1	1		2	1	1	1	1	
Interstitial Inflammation	1						1							2	2			2
Bronchitis																		
Peribronchiolar Edema																		
Alveolar Septal Collagen													1	1	1	1	1	1

EPL

Experimental Pathology Laboratories, Inc

Key P = Present
1 = Minimal
5 = Severe/High

N = No Section
2 = Slight
I = Incomplete Section

A = Autolysis
3 = Moderate

X = Not Remarkable
4 = Moderately Severe/High

HISTOPATHOLOGY INCIDENCE TABLE

Treatment Group
Exposure

PROJECT L06139
PHASE IV, STUDY NO. 80
MALE RATS
FOUR WEEK SACRIFICE

ANUM
BER

70 71 72
C0 C2 C3

	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	852
LUNG	1	1															
Hemorrhage	1	1													1	1	
Atelectasis	1	1		1	2	1		1					2				1
Alveolar Macrophages	1	1		1	2	1	1	1	1	1	1	1				1	
Eosinophilic Infiltrate					1								1				
Terminal Bronchiolar Fibrosis							1	1	1	2	1	1	3	2	3	3	3
Interstitial Inflammation	1								1					1	2	1	1
Bronchitis														1	2		
Peribronchiolar Edema													2				
Alveolar Septal Collagen							1	1	1	1	1		3	2	2	2	2

X = Not Remarkable
2 = Moderately Severe/High

A = Autolysis
3 = Moderate

N = No Section
2 = Slight
1 = Incomplete Section

Key P = Present
1 = Minimal
5 = Severe/High

Experimental Pathology Laboratories, Inc.

SPONTANEOUS DEATHS AND MORIBUND SACRIFICES

[illegible]

193

Experimental Pathology Laboratories, Inc.

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$$\begin{aligned} \text{H} &= 5 \\ \text{P} &= 1 \\ \text{S} &= 4 \end{aligned}$$

N = No Section
Z = Slight
I = Incomplete Section

$\frac{d}{dt} \left(\frac{\partial L}{\partial \dot{x}} \right) = \frac{\partial L}{\partial x}$

$\text{CH}_3\text{COOH} + \text{H}_2\text{O} = \text{X}$

IITRI PROJECT NUMBER L06139
PHASE IV STUDY NO. 80
THE EFFECTS OF SUBCHRONIC EXPOSURES TO
RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
ON VARIOUS BIOLOGICAL ENDPOINTS
IN MALE SPRAGUE-DAWLEY RATS

PATHOLOGY REPORT

TERMINAL SACRIFICE, RECOVERY SACRIFICE AND
MORTALITIES DAYS 51-125

Submitted To:

IIT Research Institute
10 West 35th Street
Chicago, IL 60616

Submitted By:

Experimental Pathology Laboratories, Inc.
Midwest Laboratory
1800 E. Pershing Road
Decatur, IL 62526

March 4, 1985

**QUALITY ASSURANCE
REPORT CERTIFICATION**

Client Name: IIT Research Institute

Client Study No.: L06139, Phase IV, Study No. 80

Study Director: Dr. W.O. Iverson

Pathologist: Dr. W.O. Iverson

Study Title: The Effects of Subchronic Exposures to Red Phosphorus/Butyl Rubber (RP/BR) Combustion Products on Various Biological Endpoints in Male Sprague-Dawley Rats

Test Article: Combustion Products of Red Phosphorus/Butyl Rubber

Species: Sprague-Dawley Rat

All parts of the pathology phase of this study, including the final report, were reviewed by Experimental Pathology Laboratories Quality Assurance Unit on February 21, 27, and March 4, 1985. All findings were reported to the Study Director and Management.


Betty L. Plankenhorn

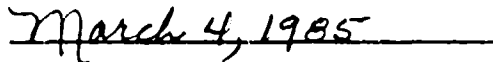

March 4, 1985

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PATHOLOGY SUMMARY

IIT PROJECT NUMBER L06139
 PHASE IV STUDY NO. 80
 THE EFFECTS OF SUBCHRONIC EXPOSURES TO
 RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
 ON VARIOUS BIOLOGICAL ENDPOINTS
 IN MALE SPRAGUE-DAWLEY RATS

PATHOLOGY SUMMARY

TERMINAL SACRIFICE, RECOVERY SACRIFICE,
 AND MORTALITIES DAYS 51-125

Microscopic examinations were performed on selected tissues from male Sprague-Dawley rats. The purpose of this study was to evaluate the effects of exposure concentration and recovery time of the repeated exposure of rats to combustion products of Red Phosphorus/Butyl Rubber (RP/BR) on various biological endpoints. This report contains the histopathologic findings for the terminal sacrifices, recovery sacrifices, and the animals which died spontaneously between 51 and 125 days on test. The experimental design for this study was as follows:

T.G. NO.	START CODE	ENDPT. GROUP	EXPO. CONC.	DOSE (mg/l)	RECOV.	EXPOSURE START	EXPOSURE END	ASSAY DATE	NO. OF ANIMALS
41	I	PATH	C0	0.0	-	8/13	11/8	11/9	12
42	I	PATH	C1	0.3	-	8/13	11/8	11/9	12
43	I	PATH	C2	0.75	-	8/13	11/8	11/9	12
44	I	PATH	C3	1.2	-	8/13	11/8	11/9	12
49	I-R	PATH	C0	0.0	+	8/13	11/8	1/4	12
50	I-R	PATH	C2	0.75	+	8/13	11/8	1/4	12
51	I-R	PATH	C3	1.2	+	8/13	11/8	1/4	12

T.G. NO. = Treatment group number

All animals were exposed for 2.25 hr/day on four consecutive days per week.

The animals sacrificed on 11/9 were designated the terminal sacrifice. Animals sacrificed on 1/4 were designated the recovery sacrifice. Total days on test were calculated for each animal with the exposure start day counted as day one. The day of death was counted as the last day on test. Actual

exposure days were always less than the days on test since the animals were only exposed four days each week. Some animals were substituted into the above treatment groups from other groups to replace animals which died spontaneously. These animals had received exposures equivalent to the animals which they replaced.

Complete gross necropsies of all rats designated for pathology were conducted. The following tissues were processed to paraffin blocks for all animals which died or were sacrificed and designated for pathology: two levels of nasal turbinates, trachea, larynx, pulmonary lymph node and lung. Additional tissues processed to paraffin blocks for the sacrificed C0 and C3 animals were heart, eye, kidney, adrenal, liver, esophagus, stomach, duodenum, urinary bladder and testis. All paraffin blocks were then shipped to Experimental Pathology Laboratories, Inc. where hematoxylin and eosin stained slides of the appropriate tissues were prepared and examined. Additional lung sections from selected animals were stained with Masson's trichrome stain to demonstrate collagen or Von Kossa's stain to demonstrate calcium salts.

RESULTS

The microscopic changes and a detailed listing of all tissues evaluated are presented in the Tabulated Animal Data Tables. Results of the special stains performed are listed in the Histopathology Incidence Table. All lesions are summarized by treatment group and presented in the Project Summary Tables. A correlation of lesions observed at necropsy with the corresponding microscopic observation, where possible, is presented in the Correlation of Gross and Micro Tables. The gross observations in these tables were transcribed from the necropsy sheets provided with the paraffin blocks. Animal dispositions were listed as final sacrifice or spontaneous death as they were indicated on the necropsy sheets.

There were no treatment-related changes found in any of the tissues which were examined outside of the respiratory tract. Lesions in general were infrequent and occurred in approximately the same incidence in C3 animals as in C0 animals.

The primary treatment-related change seen histologically in this study was in the lung and was diagnosed as "terminal bronchiolar fibrosis". The lesion consisted of thickening of the alveolar walls and of the most distal portions of the terminal bronchioles at the point where the terminal bronchiole ends and joins the alveolar sacs. The thickening consisted of a heterogenous eosinophilic material containing small numbers of cells. This material stained positively for collagen with Masson's trichrome stain. The incidence of this lesion, by severity, is shown in the following table:

INCIDENCE OF TERMINAL BRONCHIOLAR FIBROSIS

Severity	Group:	Number of Animals						
		Terminal Sac.				Recovery Sac.		
		C0	C1	C2	C3	C0	C2	C3
Minimal - Grade 1			4	3	1	5		
Mild - Grade 2				9	1	7		3
Moderate - Grade 3					6			7
Severe - Grade 4					4			2

A crystalline, basophilic character to the fibrous tissue was seen in some animals on the H&E sections. Staining with Von Kossa's method for calcium salts gave a strongly positive reaction around many of the terminal bronchioles in the three animals selected for staining. The staining was even more extensive than with the Masson's trichrome stain. The incidence of fibrosis in the recovery sacrifice animals was only slightly less than that of the terminal sacrifice animals and does not suggest resolution of the lesion with time.

Six animals which died spontaneously during the study had their respiratory tracts examined microscopically. All four animals which had been exposed to RP/BR had terminal bronchiolar fibrosis. The three C3 animals had mild to severe erosions of the laryngeal mucosa with deposition of fibrin on the surface. These laryngeal changes were probably contributory to the death of these three animals.

CONCLUSION

The results of these microscopic examinations indicate that the exposure of male Sprague-Dawley rats to the combustion products of RP/BR for 2.25 hr/day for four consecutive days per week produced terminal bronchiolar fibrosis in 100% of the animals after three months of exposure at both 0.75 and 1.2 mg/l. The severity of the lesion increases with the higher dose. Approximately 30% of the animals exposed to 0.3 mg/l for three months under similar conditions developed minimal terminal bronchiolar fibrosis. The lesion was demonstrated to contain both increased amounts of collagen as well as calcium salts. Three animals exposed to 1.2 mg/l died during the study with erosions and/or inflammation of the laryngeal mucosa which is considered treatment-related. No other changes found in the other tissues examined appeared treatment-related.

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March 4, 1985

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Reports Code Table

N	Tissues within normal histological limits
A	Autolysis precluding adequate evaluation
U	Tissues unsuitable for complete evaluation
S	Tissues not applicable to animal
*	Tissues unavailable for evaluation

1	minimal
2	mild
3	moderate
4	severe
)	focal
J	locally extensive
>	multifocal
P	Present
B	Neoplasm, Benign
M	Neoplasm, Malignant without Metastasis
C	Neoplasm, Malignant with Metastasis
X	Metastatic Site (+)

ABBREVIATION LIST

Fibro - Fibrosis

LN - Lymph Node

Lympho - Lymphocytic

MAN - Mandibular

PROJECT SUMMARY TABLES
TERMINAL SACRIFICE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Project Summary Table

SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

FATES: FINAL SACRIFICE

DAYS: 89-89

SEX: MALE

GROUP:

NUMBER OF ANIMALS:

C0

C1

C2

C3

12

12

12

12

#

%

#

%

#

%

#

%

NASAL TURBINATE-LEVEL 1

No E: 12

12

12

12

NASAL TURBINATE-LEVEL 2

No E: 12

12

12

12

TRACHEA

No E: 12

12

12

12

Erosion

0

(0)

0

(0)

0

(0)

4

(33)

LARYNX

No E: 12

12

12

12

PULMONARY LYMPH NODE

No E: 12

12

12

12

Hemorrhage

5

(42)

3

(25)

4

(33)

5

(42)

Lymphocytic Hyperplasia

10

(83)

2

(17)

1

(8)

3

(25)

Macrophage Hyperplasia

0

(0)

1

(8)

2

(17)

3

(25)

Pigment

0

(0)

1

(8)

1

(8)

0

(0)

Edema

0

(0)

1

(8)

0

(0)

0

(0)

Lymphocytic Infiltrate

0

(0)

0

(0)

0

(0)

1

(8)

LUNG

No E: 12

12

12

12

Atelectasis

5

(42)

4

(33)

1

(8)

5

(42)

Focal Lymphocyte Aggregate

0

(0)

2

(17)

1

(8)

0

(0)

Alveolar Macrophages

8

(67)

2

(17)

1

(8)

1

(8)

Interstitial Inflammation

7

(58)

1

(8)

1

(8)

0

(0)

Terminal Bronchiolar Fibro

0

(0)

4

(33)

12

(100)

12

(100)

Mineralization

0

(0)

0

(0)

0

(0)

7

(58)

HEART

No E: 12

0

0

12

Inflammation

2

(17)

0

0

0

0

(0)

Lymphocytic Infiltrate

1

(8)

0

0

0

2

(17)

EYE

No E: 12

0

0

12

Corneal Anomaly

1

(8)

0

0

0

0

(0)

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Project Summary Table
SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

DATES: FINAL SACRIFICE

DAYS: 89-89

SEX: MALE

GROUP:	C0	C1	C2	C3
NUMBER OF ANIMALS:	12	12	12	12
	#	%	#	%
KIDNEY	No Ex 12	0	0	12
Hyaline Casts	10 (83)	0	0	8 (67)
Tubular Hyperplasia	9 (75)	0	0	8 (67)
Pigment	1 (8)	0	0	2 (17)
ADRENAL	No Ex 12	0	0	12
LIVER	No Ex 12	0	0	12
Inflammation	1 (8)	0	0	1 (8)
Lymphocytic Infiltrate	6 (50)	0	0	6 (50)
ESOPHAGUS	No Ex 12	0	0	12
STOMACH	No Ex 12	0	0	12
Exfoliated Cells	1 (8)	0	0	2 (17)
DUODENUM	No Ex 12	0	0	12
URINARY BLADDER	No Ex 12	0	0	12
Concretion	1 (8)	0	0	0 (0)
Lymphocytic Infiltrate	0 (0)	0	0	1 (8)
TESTIS	No Ex 12	0	0	12

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Project Summary Table

SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

DATES: FINAL SACRIFICE

DAYS: 89-89

SEX: MALE

GROUP:	C0	C1	C2	C3
NUMBER OF ANIMALS:	12	12	12	12

OTHER TISSUES AND LESIONS:

	#	%	#	%	#	%	#	%
MAN LN-Macrophage Hyperplasia	1	(8)	1	(8)	1	(8)	0	(0)
MAN LN-Lympho Hyperplasia	1	(8)	0	(0)	1	(8)	0	(0)
MAN LN-Hemorrhage	1	(8)	1	(8)	1	(8)	0	(0)
THYMUS-Hemorrhage	1	(8)	0	(0)	1	(8)	0	(0)

PROJECT SUMMARY TABLES

RECOVERY SACRIFICE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Project Summary Table

SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

DATES: FINAL SACRIFICE

DAYS: 145-145

SEX: MALE

GROUP:

NUMBER OF ANIMALS:

C0	C1	C2	C3
12	0	12	12

	#	%	#	%	#	%	#	%
NASAL TURBINATE-LEVEL 1	No E: 12		0		12		12	
Hemorrhage	0	(0)	0		0	(0)	2	(17)
Autolysis	0	(0)	0		1	(8)	0	(0)
Eosinophilic Globules	0	(0)	0		1	(8)	0	(0)
NASAL TURBINATE-LEVEL 2	No E: 12		0		12		12	
Hemorrhage	3	(25)	0		0	(0)	3	(25)
Autolysis	0	(0)	0		1	(8)	0	(0)
TRACHEA	No E: 12		0		12		12	
Autolysis	0	(0)	0		1	(8)	0	(0)
LARYNX	No E: 12		0		12		12	
Mineralization	0	(0)	0		0	(0)	1	(8)
Autolysis	0	(0)	0		1	(8)	0	(0)
PULMONARY LYMPH NODE	No E: 12		0		12		12	
Hemorrhage	5	(42)	0		5	(42)	7	(58)
Lymphocytic Hyperplasia	7	(58)	0		3	(25)	6	(50)
Macrophage Hyperplasia	0	(0)	0		1	(8)	3	(25)
Pigment	0	(0)	0		2	(17)	0	(0)
Edema	5	(42)	0		5	(42)	4	(33)
Lymphocytic Infiltrate	1	(8)	0		0	(0)	0	(0)
Autolysis	0	(0)	0		1	(8)	0	(0)

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Project Summary Table

SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

FATES: FINAL SACRIFICE

DAYS: 145-145

SEX: MALE

GROUP:	C0	C1	C2	C3
NUMBER OF ANIMALS:	12	0	12	12
	#	%	#	%
LUNG	No Ex: 12	0	12	12
Atelectasis	6 (50)	0	3 (25)	2 (17)
Hemorrhage	1 (8)	0	2 (17)	1 (8)
Focal Lymphocyte Aggregate	1 (8)	0	1 (8)	0 (0)
Alveolar Macrophages	8 (67)	0	4 (33)	0 (0)
Interstitial Inflammation	5 (42)	0	1 (8)	0 (0)
Terminal Bronchiolar Fibro	0 (0)	0	12 (100)	12 (100)
Eosinophilic Infiltrate	2 (17)	0	1 (8)	0 (0)
Mineralization	0 (0)	0	0 (0)	6 (50)
Autolysis	2 (17)	0	1 (8)	0 (0)
HEART	No Ex: 12	0	1	12
Lymphocytic Infiltrate	4 (33)	0	0 (0)	0 (0)
EYE	No Ex: 12	0	0	12
KIDNEY	No Ex: 12	0	0	12
Hyaline Casts	11 (92)	0	0	10 (83)
Tubular Hyperplasia	10 (83)	0	0	9 (75)
Lymphocytic Infiltrate	0 (0)	0	0	1 (8)
ADRENAL	No Ex: 12	0	0	12
Accessory Cortical Tissue	0 (0)	0	0	3 (25)
LIVER	No Ex: 12	0	0	12
Inflammation	0 (0)	0	0	2 (17)
Lymphocytic Infiltrate	1 (8)	0	0	3 (25)
ESOPHAGUS	No Ex: 12	0	0	12

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Project Summary Table

SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

DATES: FINAL SACRIFICE

DAYS: 145-145

SEX: MALE

GROUP:

NUMBER OF ANIMALS:

C0

12

C1

0

C2

12

C3

12

	#	%	#	%	#	%	#	%
STOMACH	No Ex	12	0		0		12	
Exfoliated Cells	1	(8)	0		0		1	(8)
Cystic Glands	1	(8)	0		0		1	(8)
DUODENUM	No Ex	12	0		0		12	
URINARY BLADDER	No Ex	12	0		0		12	
Concretion	1	(8)	0		0		1	(8)
TESTIS	No Ex	12	0		0		12	

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Project Summary Table

SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

DATES: FINAL SACRIFICE

DAYS: 145-145

SEX: MALE

GROUP:	C0	C1	C2	C3
NUMBER OF ANIMALS:	12	0	12	12

	#	%	#	%	#	%	#	%
--	---	---	---	---	---	---	---	---

OTHER TISSUES AND LESIONS:

PREPUTIAL GLAND-Inflammation	0	(0)	0	(0)	0	(0)	1	(8)
FAT-Inflammation	0	(0)	0	(0)	1	(8)	0	(0)

PROJECT SUMMARY TABLES
SPONTANEOUS DEATHS

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/ETHYL RUBBER
(RP/ER) COMBUSTION PRODUCTS

Project Summary Table

SUMMARY: Incidence of NEOPLASTIC and NON-NEOPLASTIC Microscopic Findings

PROJECT ID. NO: 221-010

DATES: SPONTANEOUS DEATH

DAYS: 51-125

SEX: MALE

GROUP:

NUMBER OF ANIMALS:

C0

C1

C2

C3

3

0

1

3

		#	%	#	%	#	%	#	%
NASAL TURBINATE-LEVEL 1	No Ex	3		0		1		3	
NASAL TURBINATE-LEVEL 2	No Ex	3		0		1		3	
Autolysis		1 (50)		0		0 (0)		0 (0)	
TRACHEA	No Ex	3		0		1		3	
Autolysis		2 (100)		0		1 (100)		0 (0)	
LARYNX	No Ex	3		0		1		3	
Autolysis		2 (100)		0		1 (100)		0 (0)	
Erosion		0 (0)		0		0 (0)		3 (100)	
Inflammation		0 (0)		0		0 (0)		1 (33)	
PULMONARY LYMPH NODE	No Ex	3		0		1		3	
Hemorrhage		2 (100)		0		1 (100)		3 (100)	
Pigment		0 (0)		0		1 (100)		1 (33)	
Edema		0 (0)		0		0 (0)		1 (33)	
LUNG	No Ex	3		0		1		3	
Hemorrhage		1 (50)		0		1 (100)		2 (67)	
Alveolar Macrophages		1 (50)		0		0 (0)		1 (33)	
Terminal Bronchiolar Fibro		0 (0)		0		1 (100)		3 (100)	
Congestion		2 (100)		0		1 (100)		3 (100)	
Autolysis		1 (50)		0		0 (0)		0 (0)	
HEART	No Ex	0		0		0		0	
EYE	No Ex	0		0		0		0	

TABULATED ANIMAL DATA
TERMINAL SACRIFICE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	529	530	531	532	533	534	535	536	537	538
NASAL TURBINATE-LEVEL 1	N	N	N	N	N	N	N	N	N	N
NASAL TURBINATE-LEVEL 2	N	N	N	N	N	N	N	N	N	N
TRACHEA	N	N	N	N	N	N	N	N	N	N
LARYNX	N	N	N	N	N	N	N	N	N	N
PULMONARY LYMPH NODE					N				N	
Hemorrhage	-	-	-	-	-	1	2	1	-	-
Lymphocytic Hyperplasia	1	1	2	1	-	1	1	2	-	2
LUNG										
Atelectasis	3	-	-	-	1	1	-	-	1	1
Alveolar Macrophages	-	1	1	1	1	-	1	1	-	2
Interstitial Inflammation	-	2	2	-	1	-	1	1	-	2
HEART	N	N	N		N	N			N	N
Inflammation	-	-	-	-	-	-	1	1	-	-
Lymphocytic Infiltrate	-	-	-	1	-	-	-	-	-	-
EYE	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(PP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	529	530	531	532	533	534	535	536	537	538
KIDNEY		N								
Hyaline Casts	1	-	1	1	1	1	1	1	1	1
Tubular Hyperplasia	-	-	1	1	1	1	-	1	1	1
Pigment	-	-	-	-	-	-	-	1	-	-
ADRENAL	N	N	N	N	N	N	N	N	N	N
LIVER	N		N		N	N			N	
Inflammation	-	1	-	-	-	-	-	-	-	-
Lymphocytic Infiltrate	-	-	-	1	-	-	1	1	-	1
ESOPHAGUS	N	N	N	N	N	N	N	N	N	N
STOMACH	N	N	N	N	N	N	N	N		N
Exfoliated Cells	-	-	-	-	-	-	-	-	2	-
DUODENUM	N	N	N	N	N	N	N	N	N	N
URINARY BLADDER	N	N	N	N	N	N	N	N	N	N
TESTIS	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	529	530	531	532	533	534	535	536	537	538
OTHER TISSUES AND LESIONS:										
MAN LN-Macrophage Hyperplasia	-	-	-	-	-	2	-	-	-	-
MAN LN-Lympho hyperplasia	-	-	-	-	-	2	-	-	-	-
MAN LN-Hemorrhage	-	-	-	-	-	2	-	-	-	-
THYMUS-Hemorrhage	-	-	-	2	-	-	-	-	-	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	539	540
NASAL TURBINATE-LEVEL 1	N	N
NASAL TURBINATE-LEVEL 2	N	N
TRACHEA	N	N
LARYNX	N	N
PULMONARY LYMPH NODE		
Hemorrhage	1	1
Lymphocytic Hyperplasia	1	1
LUNG		N
Alveolar Macrophages	2	-
Interstitial Inflammation	2	-
HEART	N	N
EYE		N
Corneal Anomaly	P	-
KIDNEY		
Hyaline Casts	1	-
Tubular Hyperplasia	1	1

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	539	540
ADRENAL	N	N
LIVER		
Lymphocytic Infiltrate	1	1
ESOPHAGUS	N	N
STOMACH	N	N
DUODENUM	N	N
URINARY BLADDER	N	
Concretion	-	P
TESTIS	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C1 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	260	541	542	543	544	545	546	547	548	549
NASAL TURBINATE-LEVEL 1	N	N	N	N	N	N	N	N	N	N
NASAL TURBINATE-LEVEL 2	N	N	N	N	N	N	N	N	N	N
TRACHEA	N	N	N	N	N	N	N	N	N	N
LARYNX	N	N	N	N	N	N	N	N	N	N
PULMONARY LYMPH NODE	N	N	N	N		N	N	N		N
Hemorrhage	-	-	-	-	2	-	-	-	-	-
Lymphocytic Hyperplasia	-	-	-	-	2	-	-	-	2	-
Pigment	-	-	-	-	-	-	-	-	1	-
LUNG	N	N			N	N		N		
Atelectasis	-	-	-	1	-	-	1	-	-	1
Focal Lymphocyte Aggregate	-	-	1	-	-	-	-	-	1	-
Alveolar Macrophages	-	-	-	-	-	-	1	-	1	-
Interstitial Inflammation	-	-	-	-	-	-	-	-	1	-
Terminal Bronchiolar Fibro	-	-	-	-	-	-	1	-	1	1

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C1 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	260	541	542	543	544	545	546	547	548	549
OTHER ISSUES AND LESIONS:										
MAN LN-Macrophage Hyperplasia	-	-	-	-	-	-	-	1	-	-
MAN LN-Hemorrhage	-	-	-	-	-	-	-	1	-	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C1 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	551	552
NASAL TURRINATE-LEVEL 1	N	N
NASAL TURRINATE-LEVEL 2	N	N
TRACHEA	N	N
LARYNX	N	N
PULMONARY LYMPH NODE		
Hemorrhage	1	1
Macrophage Hyperplasia	-	1
Edema	1	-
LUNG	N	
Atelectasis	-	1
Terminal Bronchiolar Fibro	-	1

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/RUTYL RUBBER
(RP/RR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CC SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	553	554	555	556	557	558	559	560	561	562
NASAL TURBINATE-LEVEL 1	N	N	N	N	N	N	N	N	N	N
NASAL TURBINATE-LEVEL 2	N	N	N	N	N	N	N	N	N	N
TRACHEA	N	N	N	N	N	N	N	N	N	N
LARYNX	N	N	N	N	N	N	N	N	N	N
PULMONARY LYMPH NODE		N	N		N	N	N			
Hemorrhage	3	-	-	-	-	-	-	2	1	-
Lymphocytic Hyperplasia	-	-	-	-	-	-	-	-	-	1
Macrophage Hyperplasia	-	-	-	1	-	-	-	-	-	-
Pigment	2	-	-	-	-	-	-	-	-	-
LUNG										
Atelectasis	1	-	-	-	-	-	-	-	-	-
Focal Lymphocyte Aggregate	-	1	-	-	-	-	-	-	-	-
Alveolar Macrophages	-	-	-	-	-	-	-	-	-	1
Interstitial Inflammation	-	-	-	-	-	-	-	-	-	1
Terminal Bronchiolar Fibro	2	2	2	2	2	1	2	2	1	1

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	553	554	555	556	557	558	559	560	561	562
OTHER TISSUES AND LESIONS:										
MAN LN-Macrophage Hyperplasia	-	-	-	-	-	-	-	-	1	-
MAN LN-Lympho Hyperplasia	-	-	-	-	-	-	-	-	2	-
MAN LN-Hemorrhage	-	-	-	-	-	-	-	-	1	-
THYMUS-Hemorrhage	-	-	-	-	-	-	1	-	-	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CC SEX: MALE DAYS: 89-89
DATES: FINAL SACRIFICE

ANIMAL ID. NO:	563	564
NASAL TURBINATE-LEVEL 1	N	N
NASAL TURBINATE-LEVEL 2	N	N
TRACHEA	N	N
LARYNX	N	N
PULMONARY LYMPH NODE		
Hemorrhage	-	3
Macrophage Hyperplasia	2	-
LUNG		
Terminal Bronchiolar Fibro	2	2

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 231-010 GROUP: C3 SEX: MALE DAYS: 89-89
DATES: FINAL SACRIFICE

ANIMAL ID. NO:	55	565	566	567	568	569	570	572	573	574
NASAL TURBINATE-LEVEL 1	N	N	N	N	N	N	N	N	N	N
NASAL TURBINATE-LEVEL 2	N	N	N	N	N	N	N	N	N	N
TRACHEA	N	N		N	N			N		N
Erosion	-	-	1	-	-	1	1	-	2	-
LARYNX	N	N	N	N	N	N	N	N	N	N
PULMONARY LYMPH NODE			N							N
Hemorrhage	3	3	-	2	1	-	2	-	-	-
Lymphocytic Hyperplasia	1	-	-	-	-	1	-	-	-	-
Macrophage Hyperplasia	-	-	-	-	1	-	-	1	1	-
Lymphocytic Infiltrate	-	-	-	-	-	-	1	-	-	-
LUNG										
Atelectasis	-	-	-	1	-	1	-	1	-	1
Alveolar Macrophages	-	-	-	1	-	-	-	-	-	-
Terminal Bronchiolar Fibro	4	2	1	3	3	4	4	3	3	3
Mineralization	1	-	-	-	-	1	1	1	1	1
HEART	N	N	N	N			N	N	N	N
Lymphocytic Infiltrate	-	-	-	-	1	1	-	-	-	-
EYE	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	55	565	566	567	568	569	570	572	573	574
KIDNEY	N									N
Hyaline Casts	-	1	1	1	1	1	1	1	-	-
Tubular Hyperplasia	-	1	1	1	1	1	1	-	1	-
Pigment	-	-	-	-	-	-	-	1	1	-
ADRENAL	N	N	N	N	N	N	N	N	N	N
LIVER			N				N		N	N
Inflammation	-	-	-	-	1	-	-	-	-	-
Lymphocytic Infiltrate	1	1	-	1	-	1	-	1	-	-
ESOPHAGUS	N	N	N	N	N	N	N	N	N	N
STOMACH	N	N	N		N		N	N	N	N
Exfoliated Cells	-	-	-	1	-	1	-	-	-	-
DUODENUM	N	N	N	N	N	N	N	N	N	N
URINARY BLADDER	N	N	N	N	N	N	N	N		N
Lymphocytic Infiltrate	-	-	-	-	-	-	-	-	1	-
TESTIS	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	575	576
NASAL TURBINATE-LEVEL 1	N	N
NASAL TURBINATE-LEVEL 2	N	N
TRACHEA	N	N
LARYNX	N	N
PULMONARY LYMPH NODE Lymphocytic Hyperplasia	N -	 1
LUNG		
Atelectasis	1	-
Terminal Bronchiolar Fibro	3	4
Mineralization	-	1
HEART	N	N
EYE	N	N
KIDNEY	N	
Hyaline Casts	-	1
Tubular Hyperplasia	-	1
ADRENAL	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 89-89
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	575	576
LIVER	N	
Lymphocytic Infiltrate	-	1
ESOPHAGUS	N	N
STOMACH	N	N
DUODENUM	N	N
URINARY BLADDER	N	N
TESTIS	N	N

TABULATED ANIMAL DATA
RECOVERY SACRIFICE

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 145-145
DATES: FINAL SACRIFICE

ANIMAL ID. NO:	626	627	628	629	630	631	632	633	634	635
NASAL TURBINATE-LEVEL 1	N	N	N	N	N	N	N	N	N	N
NASAL TURBINATE-LEVEL 2 Hemorrhage	N	-	N	N	-	N	N	N	N	-
TRACHEA	N	N	N	N	N	N	N	N	N	N
LARYNX	N	N	N	N	N	N	N	N	N	N
PULMONARY LYMPH NODE					N	N			N	
Hemorrhage	-	1	2	-	-	-	1	1	-	2
Lymphocytic Hyperplasia	1	2	-	2	-	-	1	1	-	-
Edema	-	-	1	1	-	-	1	-	-	-
Lymphocytic Infiltrate	-	-	1	-	-	-	-	-	-	-
LUNG										
Atelectasis	-	1	-	-	1	1	1	1	-	-
Hemorrhage	-	-	1	-	-	-	-	-	-	-
Alveolar Macrophages	1	2	1	1	1	-	1	-	1	-
Interstitial Inflammation	1	1	-	1	1	-	1	-	-	-
Eosinophilic Infiltrate	-	-	-	1	1	-	-	-	-	-
Autolysis	-	1	-	-	-	-	-	-	-	1
HEART										
Lymphocytic Infiltrate	1	-	-	-	-	-	1	1	-	-
EYE	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/RR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 145-145
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	626	627	628	629	630	631	632	633	634	635
KIDNEY										
Hyaline Casts	1	1	1	1	1	1	1	1	1	1
Tubular Hyperplasia	1	1	1	1	1	1	1	1	1	1
ADRENAL	N	N	N	N	N	N	N	N	N	N
LIVER	N	N	N	N	N	N	N	N	N	N
ESOPHAGUS	N	N	N	N	N	N	N	N	N	N
STOMACH	N		N		N	N	N	N	N	N
Exfoliated Cells	-	-	-	1	-	-	-	-	-	-
Cystic Glands	-	1	-	-	-	-	-	-	-	-
DUODENUM	N	N	N	N	N	N	N	N	N	N
URINARY BLADDER		N	N	N	N	N	N	N	N	N
Concretion	P	-	-	-	-	-	-	-	-	-
TESTIS	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 321-010 GROUP: CO SEX: MALE DAYS: 145-145
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	636	661
NASAL TURBINATE-LEVEL 1	N	N
NASAL TURBINATE-LEVEL 2	N	N
TRACHEA	N	N
LARYNX	N	N
PULMONARY LYMPH NODE		
Lymphocytic Hyperplasia	1	2
Edema	2	1
LUNG		
Atelectasis	1	-
Focal Lymphocyte Aggregate	-	1
Alveolar Macrophages	-	1
HEART		N
Lymphocytic Infiltrate	1	-
EYE	N	N
KIDNEY		N
Hyaline Casts	1	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 145-145
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	636	661
ADRENAL	N	N
LIVER	N	
Lymphocytic Infiltrate	-	1
ESOPHAGUS	N	N
STOMACH	N	N
DUODENUM	N	N
URINARY BLADDER		N
TESTIS	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/RR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-G10 GROUP: C2 SEX: MALE DAYS: 145-145
DATES: FINAL SACRIFICE

ANIMAL ID. NO:	638	639	640	641	642	643	644	645	646	647
NASAL TURBINATE-LEVEL 1	N	N	N	N	N		N	N	N	N
Eosinophilic Globule(s)	-	-	-	-	-	1	-	-	-	-
NASAL TURBINATE-LEVEL 2	N	N	N	N	N	N	N	N	N	N
TRACHEA	N	N	N	N	N	N	N	N	N	N
LARYNX	N	N	N	N	N	N	N	N	N	N
PULMONARY LYMPH NODE						N				N
Hemorrhage	-	-	1	-	1	-	3	-	3	-
Lymphocytic Hyperplasia	2	1	-	-	1	-	-	-	-	-
Macrophage Hyperplasia	-	1	-	-	-	-	-	-	-	-
Pigment	-	-	-	-	-	-	2	-	2	-
Edema	-	-	-	1	1	-	1	1	-	-
LUNG										
Atelectasis	-	2	-	-	1	1	-	-	-	-
Hemorrhage	-	-	-	-	-	-	-	1	-	1
Focal Lymphocyte Aggregate	2	-	-	-	-	-	-	-	-	-
Alveolar Macrophages	2	-	1	-	-	-	-	1	1	-
Interstitial Inflammation	2	-	-	-	-	-	-	-	-	-
Terminal Bronchiolar Fibro	1	1	2	2	2	2	2	1	1	2
Eosinophilic Infiltrate	2	-	-	-	-	-	-	-	-	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 145-146
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	638	639	640	641	642	643	644	645	646	647
OTHER TISSUES AND LESIONS:										
EAT-Inflammation	-	-	-	-	-	2	-	-	-	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 145-145
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	648	673
NASAL TURBINATE-LEVEL 1		N
Autolysis	P	-
NASAL TURBINATE-LEVEL 2		N
Autolysis	P	-
TRACHEA		N
Autolysis	P	-
LARYNX		N
Autolysis	P	-
PULMONARY LYMPH NODE		
Hemorrhage	-	2
Edema	-	2
Autolysis	P	-
LUNG		
Terminal Bronchiolar Fibro	1	2
Autolysis	P	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 145-146
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	650	651	652	653	654	655	656	657	658	660
NASAL TURBINATE-LEVEL 1 Hemorrhage	N -	 1	N -	 1	N -	N -	N -	N -	N -	N -
NASAL TURBINATE-LEVEL 2 Hemorrhage	N -	N -	N -	 3	N -	 1	N -	N -	 2	N -
TRACHEA	N	N	N	N	N	N	N	N	N	N
LARYNX	N	N	N	N	N	N	N	N	N	N
PULMONARY LYMPH NODE	N									
Hemorrhage	-	-	1	2	1	3	-	1	-	1
Lymphocytic Hyperplasia	-	-	1	-	2	-	1	-	1	-
Macrophage Hyperplasia	-	-	-	-	-	1	-	-	1	-
Edema	-	1	-	1	-	-	-	-	-	-
LUNG										
Atelectasis	-	-	-	-	-	-	1	1	-	-
Hemorrhage	1	-	-	-	-	-	-	-	-	-
Terminal Bronchiolar Fibro	2	2	3	3	3	3	4	3	3	3
Mineralization	-	-	1	-	-	-	2	1	1	1
HEART	N	N	N	N	N	N	N	N	N	N
EYE	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 331-010 GROUP: C3 SEX: MALE DAYS: 145-146
DATES: FINAL SACRIFICE

ANIMAL ID. NO:	650	651	652	653	654	655	656	657	658	660
KIDNEY	N								N	
Hyaline Casts	-	1	1	1	1	1	1	1	-	1
Tubular Hyperplasia	-	1	1	1	1	-	1	1	-	1
ADRENAL	N		N	N	N		N	N		N
Accessory Cortical Tissue	-	1	-	-	-	P	-	-	P	-
LIVER	N	N	N				N		N	N
Inflammation	-	-	-	-	1	2	-	-	-	-
Lymphocytic Infiltrate	-	-	-	1	-	2	-	1	-	-
ESOPHAGUS	N	N	N	N	N	N	N	N	N	N
STOMACH	N	N			N	N	N	N	N	N
Exfoliated Cells	-	-	-	3	-	-	-	-	-	-
Cystic Glands	-	-	1	-	-	-	-	-	-	-
DUODENUM	N	N	N	N	N	N	N	N	N	N
URINARY BLADDER	N		N	N	N	N	N	N	N	N
Concretion	-	P	-	-	-	-	-	-	-	-
TESTIS	N	N	N	N	N	N	N	N	N	N

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 145-145
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	650	651	652	653	654	655	656	657	658	660
OTHER TISSUES AND LESIONS:										
PREPUTIAL GLAND-Inflammation	-	-	-	-	-	-	-	-	4	-

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 145-145
DATES: FINAL SACRIFICE

ANIMAL ID. NO:	685	692
NASAL TURBINATE-LEVEL 1	N	N
NASAL TURBINATE-LEVEL 2	N	N
TRACHEA	N	N
LARYNX	N	
Mineralization	-	1
PULMONARY LYMPH NODE		
Hemorrhage	-	2
Lymphocytic Hyperplasia	1	1
Macrophage Hyperplasia	1	-
Edema	1	2
LUNG		
Terminal Bronchiolar Fibro	4	2
Mineralization	2	-
HEART	N	N
EYE	N	N
KIDNEY		
Myaline Casts	1	1
Tubular Hyperplasia	1	1
Lymphocytic Infiltrate	1	-

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 145-145
FATES: FINAL SACRIFICE

ANIMAL ID. NO:	685	692
ADRENAL	N	N
LIVER	N	N
ESOPHAGUS	N	N
STOMACH	N	N
DUODENUM	N	N
URINARY BLADDER	N	N
TESTIS	N	N

TABULATED ANIMAL DATA

SPONTANEOUS DEATHS

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: CO SEX: MALE DAYS: 71-125
EATES: SPONTANEOUS DEATH

ANIMAL ID. NO:	85	160
NASAL TURBINATE-LEVEL 1	N	N
NASAL TURBINATE-LEVEL 2	N	
Autolysis	-	P
TRACHEA		
Autolysis	P	P
LARYNX		
Autolysis	P	P
PULMONARY LYMPH NODE		
Hemorrhage	4	1
LUNG		
Hemorrhage	-	2
Alveolar Macrophages	4	-
Congestion	4	3
Autolysis	-	P

THE EFFECTS OF SURCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 107-107
FATES: SPONTANEOUS DEATH

ANIMAL ID. NO:	172
NASAL TURBINATE-LEVEL 1	N
NASAL TURBINATE-LEVEL 2	N
TRACHEA Autolysis	P
LARYNX Autolysis	P
PULMONARY LYMPH NODE Hemorrhage	2
Pigment	2
LUNG Hemorrhage	2
Terminal Bronchiolar Fibro	2
Congestion	2

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Tabulated Animal Data

PROJECT IS. 221-010 GROUP: C3 SEX: MALE DAYS: 51-78
FATES: SPONTANEOUS DEATH

ANIMAL ID. NO:	135	186	331
NASAL TURBINATE-LEVEL 1	N	N	N
NASAL TURBINATE-LEVEL 2	N	N	N
TRACHEA	N	N	N
LARYNX			
Erosion	2	3	4
Inflammation	-	3	-
PULMONARY LYMPH NODE			
Hemorrhage	2	3	4
Pigment	-	-	2
Edema	-	-	1
LUNG			
Hemorrhage	-	1	2
Alveolar macrophages	-	-	1
Terminal bronchiolar Fibro	2	2	2
Congestion	2	2	2

HISTOPATHOLOGY INCIDENCE TABLE
SPECIAL STAINS

HISTOPATHOLOGY INCIDENCE TABLE (SPECIAL STAINS)

PROJECT NUMBER L06139
 PHASE IV STUDY NO. 80
 SUBCHRONIC EXPOSURE TO RP/BR
 MALE SPRAGUE-DAWLEY RATS
 RECOVERY SACRIFICE

ANUM TMBL AL	EXPOSURE GROUP															
	C0	C2				C3										
LUNG	627	X														
Alveolar Septal Collagen	631	X														
Calcium Salts	638	2	2	3	2	3	4	4	4	3						
	641	N	N	N	N											
	642															
	644															
	645															
	651															
	656															
	658															
	660															
	685															

EPI 43

Key P = Present
 1 = Minimal
 5 = Severe/High

N = No Section
 2 = Slight
 I = Incomplete Section

A = Autolysis
 3 = Moderate
 4 = Moderately Severe/High
 X = Not Remarkable

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CORRELATION OF GROSS AND MICRO TABLES

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 89-89
PAGE 1
FATES: FINAL SACRIFICE

ANIMAL NO: 529 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 530 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 531 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 532 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>THYMUS: Scattered Dark Red Foci THYMUS- Hemorrhage

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 89-89
PAGE 2
FATES: FINAL SACRIFICE

ANIMAL NO: 533
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>LUNG: Left, Scattered Red Foci

NO COROLLARY CHANGE DETECTED

ANIMAL NO: 534
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>MANDIBULAR LYMPH NODE- Dk. Red

MANDIBULAR LYMPH NODE-
Hemorrhage

ANIMAL NO: 535
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 536
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

>
NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 89-89
PAGE 3
FATES: FINAL SACRIFICE

ANIMAL NO: 537 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
›NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 538 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
›LUNG: Scattered 0.1 To 0.5 cm LUNG- Interstitial Inflammation
 Gray Raised Foci
›BLADDER: Filled With Yellow Fluid NO COROLLARY CHANGE DETECTED

ANIMAL NO: 539 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
›NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 89-89
PAGE 4
FATES: FINAL SACRIFICE

ANIMAL NO: 540 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C1 SEX: MALE DAYS: 89-89
PAGE 1
FATES: FINAL SACRIFICE

ANIMAL NO: 260
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

ANIMAL NO: 541
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

ANIMAL NO: 542
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

ANIMAL NO: 543
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RF/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C1 SEX: MALE DAYS: 89-89
PAGE 2
FATES: FINAL SACRIFICE

ANIMAL NO: 544 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 545 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 546 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 547 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>MANDIBULAR LYMPH NODE- Red MANDIBULAR LYMPH NODE- Hemorrhage

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C1 SEX: MALE DAYS: 89-89
PAGE 3
FATES: FINAL SACRIFICE

ANIMAL NO: 548
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 549
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 551
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 552
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RF/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 89-89
PAGE 1
FATES: FINAL SACRIFICE

ANIMAL NO: 553
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 554
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 555
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 556
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 89-89
PAGE 2
FATES: FINAL SACRIFICE

ANIMAL NO: 557
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 558
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 559
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>THYMUS: Scattered Dark Red
Pinpoint Foci

THYMUS- Hemorrhage

ANIMAL NO: 560
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 89-89
PAGE 3
FATES: FINAL SACRIFICE

ANIMAL NO: 561 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>MANDIBULAR LYMPH NODE: Dark Red MANDIBULAR LYMPH NODE- Hemorrhage

ANIMAL NO: 562 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 563 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 564 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 89-89
PAGE 1
FATES: FINAL SACRIFICE

ANIMAL NO: 55
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

ANIMAL NO: 565
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

ANIMAL NO: 566
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

ANIMAL NO: 567
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:
>NO OBSERVABLE ABNORMALITIES.

RELATED HISTOPATHOLOGY:
NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 89-89
PAGE 2
FATES: FINAL SACRIFICE

ANIMAL NO: 568 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 569 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 570 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 572 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
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Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 89-89
PAGE 3
FATES: FINAL SACRIFICE

ANIMAL NO: 573 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 574 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>LUNG: Mottled, Grayish LUNG- Terminal Bronchiolar
Fibrosis

ANIMAL NO: 575 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 576 PATHOLOGIST: WOI
DAYS ON TEST: 89
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 145-145
PAGE 1
FATES: FINAL SACRIFICE

ANIMAL NO: 626
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

URINARY BLADDER: Calculi In
Bladder

URINARY BLADDER- Concretion

ANIMAL NO: 627
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 628
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 629
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
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(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 145-145
PAGE 2
FATES: FINAL SACRIFICE

ANIMAL NO: 630 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 631 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 632 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 633 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 145-145
PAGE 3
FATES: FINAL SACRIFICE

ANIMAL NO: 634 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 635 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 636 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 661 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 145-145
PAGE 1
FATES: FINAL SACRIFICE

ANIMAL NO: 638 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 639 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>LUNG: Right Posterior, (Small NO COROLLARY CHANGE DETECTED
 Foci)
>LUNG: Left Lung, (Small Foci) NO COROLLARY CHANGE DETECTED

ANIMAL NO: 640 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 145-145
PAGE 2
FATES: FINAL SACRIFICE

ANIMAL NO: 641 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 642 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 643 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >FAT: Abdominal Necrotic Fat FAT- Inflammation
 Body, 0.2 x 0.2 x 0.1 cm

ANIMAL NO: 644 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >TESTIS: Right, Small, 0.3 x 0.2 No Section
 x 0.1 cm

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 145-145
PAGE 3
FATES: FINAL SACRIFICE

ANIMAL NO: 645 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 646 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 647 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 648 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
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RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 145-145
PAGE 4
FATES: FINAL SACRIFICE

ANIMAL NO: 673 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 145-145
PAGE 1
FATES: FINAL SACRIFICE

ANIMAL NO: 650 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 651 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 652 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 653 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
 >NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 145-145
PAGE 2
FATES: FINAL SACRIFICE

ANIMAL NO: 654
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>NO OBSERVABLE ABNORMALITIES.

NOT APPLICABLE

ANIMAL NO: 655
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>KIDNEY: Flaccid

NO COROLLARY CHANGE DETECTED

ANIMAL NO: 656
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>URINARY BLADDER: Yellow Fluid In
Bladder

NO COROLLARY CHANGE DETECTED

ANIMAL NO: 657
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>KIDNEY: Dilated Pelvis; Flaccid

NO COROLLARY CHANGE DETECTED

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS
RECOVERY SACRIFICE GROUP

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 145-145
PAGE 3
FATES: FINAL SACRIFICE

ANIMAL NO: 658 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>PREPUTIAL GLAND: Abscess PREPUTIAL GLAND- Inflammation

ANIMAL NO: 660 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 685 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

ANIMAL NO: 692 PATHOLOGIST: WOI
DAYS ON TEST: 145
ANIMAL FATE: FINAL SACRIFICE

REFERENCE TO NECROPSY RECORD: RELATED HISTOPATHOLOGY:
>NO OBSERVABLE ABNORMALITIES. NOT APPLICABLE

THE EFFECTS OF SUBCHRONIC EXPOSURES
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Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 71-125
PAGE 1
FATES: SPONTANEOUS DEATH

ANIMAL NO: 85
DAYS ON TEST: 71
ANIMAL FATE: SPONTANEOUS DEATH

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:	RELATED HISTOPATHOLOGY:
>HEART: Enlarged; Firm	No Section
>LUNG: Dark Red	LUNG- Congestion
>LIVER: Dark Red	No Section
>ADRENAL: Enlarged, 0.4 x 0.4 cm	No Section
>JEJUNUM: Moderately Autolysed	No Section
>ILEUM: Moderately Autolysed	No Section

ANIMAL NO: 160
DAYS ON TEST: 125
ANIMAL FATE: SPONTANEOUS DEATH

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:	RELATED HISTOPATHOLOGY:
>MANDIBULAR SALIVARY GLAND: Dark Red	No Section
>MANDIBULAR LYMPH NODE: Dark Red	No Section
>THYMUS: Mottled Dark Red	No Section
>LUNG: Mottled Dark Red	LUNG- Congestion
>LIVER: Dark Red	No Section
>KIDNEY: Mottled Dark Red	No Section
>ADRENAL: Red	No Section
>SPLEEN: Dark Red	No Section

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C0 SEX: MALE DAYS: 71-125
PAGE 2
FATES: SPONTANEOUS DEATH

ANIMAL NO: 160
DAYS ON TEST: 125
ANIMAL FATE: SPONTANEOUS DEATH

PATHOLOGIST: WOI

REFERENCE TO NECROPSY RECORD:

RELATED HISTOPATHOLOGY:

>INTESTINAL TRACT: Slightly Autolysed	No Section
>CECUM: Gas Filled	No Section
>MESENTERIC LYMPH NODE: Dark Red	No Section
>BRAIN: Slightly Autolysed	No Section

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
(RP/BR) COMBUSTION PRODUCTS

Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C2 SEX: MALE DAYS: 107-107
PAGE 1
FATES: SPONTANEOUS DEATH

ANIMAL NO: 172 PATHOLOGIST: WOI
DAYS ON TEST: 107
ANIMAL FATE: SPONTANEOUS DEATH

REFERENCE TO NECROPSY RECORD:	RELATED HISTOPATHOLOGY:
>THORACIC CAVITY: Pool Of Dark Red Fluid	No Section
>LUNG: Multiple Dark Red Areas Especially The Posterior Ends Of The Right Posterior And Left Lobes	LUNG- Hemorrhage
>HEART: Very Firm	No Section
>KIDNEY: Softer	No Section
>URINARY BLADDER: Contained Calculi	No Section
>CECUM: Moderately Bloated	No Section

THE EFFECTS OF SUBCHRONIC EXPOSURES
TO RED PHOSPHORUS/BUTYL RUBBER
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Correlation of Gross & Micro

PROJECT ID: 221-010 GROUP: C3 SEX: MALE DAYS: 51-78
PAGE 1
FATES: SPONTANEOUS DEATH

ANIMAL NO: 135 PATHOLOGIST: WOI
DAYS ON TEST: 51
ANIMAL FATE: SPONTANEOUS DEATH

REFERENCE TO NECROPSY RECORD:	RELATED HISTOPATHOLOGY:
>LUNG: Mottled Dark Red	LUNG- Congestion
>LIVER: Dark Red	No Section

ANIMAL NO: 186 PATHOLOGIST: WOI
DAYS ON TEST: 78
ANIMAL FATE: SPONTANEOUS DEATH

REFERENCE TO NECROPSY RECORD:	RELATED HISTOPATHOLOGY:
>LUNG: Mottled Red	LUNG- Congestion
>LIVER: Dark Red	No Section
>STOMACH: Distended And Gas Filled	No Section

ANIMAL NO: 331 PATHOLOGIST: WOI
DAYS ON TEST: 59
ANIMAL FATE: SPONTANEOUS DEATH

REFERENCE TO NECROPSY RECORD:	RELATED HISTOPATHOLOGY:
>LUNG: Moderately Dark Red	LUNG- Congestion
>LIVER: Dark Red	No Section
>HEART: Firm	No Section

IIT PROJECT NUMBER 106139
PHASE IV STUDY NO. 80
THE EFFECTS OF SUBCHRONIC EXPOSURES TO
RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
ON VARIOUS BIOLOGICAL ENDPOINTS
IN MALE SPRAGUE-DAWLEY RATS

PATHOLOGY REPORT
ADDENDUM

Pneumonia virus of mice (PVM) is a pneumovirus first described in 1940. Recent studies have shown that it is of low transmissability, even among animals housed together, and extremely labile in the environment. Most laboratory rodents, including rats, are susceptible. The characteristic lesions associated with the virus are an acute vasculitis which develops into patchy interstitial pneumonia. Perivascular and peribronchiolar accumulation of lymphocytes are also present. Fibrosis at the terminal bronchiole, which is the primary effect of RP/BR exposure, has not been reported with PVM infections. Therefore, the lesions produced by PVM infection are separate and distinct from those which were treatment-related.

The combination of lesions produced by PVM was also not present in the twenty-two rats which died spontaneously during the study. Their death was not due to PVM infection. Congestion and hemorrhage of the lung were the most common findings in these animals. The cause of death in most of these cases is not known but exposure to RP/BR may have been a contributing factor.

Three animals which died spontaneously after day 51 had erosions of the laryngeal mucosa with deposits of fibrin on the surface. This fibrin probably partially obstructed the larynx and may have contributed to the death of these three animals. They were all in Group C3 and the lesion is considered related to RP/BR exposure.

Best Available Copy

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W.O. Iverson, D.V.M.
Diplomate, ACVP
July 29, 1986

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September 17, 1985

Ms. Catherine Aranyi
IIT Research Institute
10 West 35th Street
Chicago, IL 60616

Dear Catherine:

Please find enclosed a recent article about the Von Kossa stain we used on some of the lung sections for your Project Number L06139. Von Kossa is apparently not specific for calcium, but will stain phosphates and carbonates or organic material in general. I suggest that we do some staining with Alizarin Red S which is specific for calcium on our next batch of lung tissue. We will work up a per slide cost for you if you would like. It would, of course, be done on a selective basis.

Best Regards,



W.O. Iverson, D.V.M.

WOI/jcs
Enclosure

Chemical Mechanisms of Staining Methods

Von Kossa's Technique: What von Kossa Really Wrote and a Modified Reaction for Selective Demonstration of Inorganic Phosphates

Susan N. Meloan, HT (ASCP), and Holde Puchtler, M.D.

Abstract

It is generally believed that in von Kossa's technique silver cations react with phosphates and carbonates in calcium deposits and are then reduced to black metallic silver by strong light. Perusal of von Kossa's paper showed that he was aware of significant differences between reactions of silver phosphate *in vitro* and in calcium deposits. He regarded only the yellow coloration of calcium deposits during early stages of the reaction as diagnostic for calcium phosphate and ascribed the blackening to organic matter. Efforts to prevent this blackening were unsuccessful. Von Kossa's experiments were reproducible in our hands. Further studies showed that bright light, generally regarded as essential for von Kossa's reaction, only causes the irreversible blackening of organic matter that masks the yellow silver phosphate. When the reaction is performed in subdued light, yellow to yellowish brown silver phosphate is visualized selectively. Silver carbonate dissolves in thiosulfate and cannot be demonstrated with von Kossa's technique.

Introduction

In discussions of von Kossa's technique, it is usually stated that silver cations react with phosphates and carbonates in calcium deposits and are then reduced to black metallic silver by light or a photographic developer. During histochemical studies of this reaction,¹ it became clear that von Kossa's² concepts have not been rendered correctly in recent histochemical and histological treatises. In contrast to current hypotheses, von Kossa's² interpretation of his observations was based on chemical data and contemporary silver techniques. Since this knowledge is no longer readily available, major findings will be reviewed briefly.

Terminology — The term "calcium deposits" will be used in the morphologically descriptive sense without any chemical implications. Such deposits are known to contain organic matter and probably cations other than calcium.

Historical Review

Early chemists knew that alkali carbonates and organic compounds reduce silver nitrate to black metallic silver, but silver phosphate remains yellow.³ During much of the 19th century silver nitrate was prescribed for internal use in the treatment of various diseases. The reduction of ingested silver to metallic silver caused gray to bluish-black discoloration (argyria) of skin.⁴⁻⁶ Light was not necessary for reduction. Virchow⁶ described blue-black coloration of renal glomeruli and interstitial collagen. Riemer⁷ found black silvered connective tissue fibers in many organs and arteries, except in central nervous tissue.

In 1844 silver nitrate was introduced into histology for blackening of cell borders of epithelium.⁸ This technique is still used in arteriosclerosis research for visualization of cell borders of endothelial cells, though it has been the subject of much controversy since the 1860's. Harpeck,⁹ Hartmann¹⁰ and Schweigger-Seidel¹¹ regarded the supposed cell borders as artifacts; similar patterns were obtained on substrates that lacked cells, e.g., collodium films.¹²⁻¹⁴ Histochemical studies showed that silver was reduced by proteins and alkali chlorides in tissue sections.^{10-13,16} This process could be speeded up by exposure to light.¹¹⁻¹³ These studies were carried out on fresh tissues; fixation and other reagents interfered with the reduction of silver nitrate.⁸

Von Kossa's Studies

Von Kossa² investigated experimentally induced calcifications in kidneys. Chemical analyses demonstrated calcium phosphate in such lesions, but required

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destruction of tissues. Special stains for calcium were not yet available. Von Kossa, therefore, searched for a histochemical technique. In *in vitro* experiments, silver nitrate solutions colored calcium phosphate crystals yellow without dissolving them. Von Kossa² then treated tissue sections with silver nitrate and obtained yellow coloration of deposits. Further chemical tests proved that the reactive material was indeed calcium phosphate. However, the yellow color in tissue sections was not permanent, but changed to gray and black. In this respect, calcium deposits in tissues differed from calcium phosphates in *in vitro* experiments: silver phosphate formed in the latter retained its bright yellow color for days and was not reduced by exposure to light. *In vitro* blackening could be obtained by addition of egg albumen or solutions containing proteins. Von Kossa², therefore, concluded that calcium deposits in tissues contain organic material that can reduce silver. However, he regarded only the yellow coloration, observed in early stages of the reaction, diagnostic for calcium phosphate. This precise chemical distinction was overlooked by later authors and has apparently been lost from current literature.

Clearly, von Kossa's² technique is a two-step reaction. In the first step, silver cations react with components of calcium deposits. In the second step, bound silver is reduced to black metallic silver by organic material with the aid of light or by photographic developers. Traditional histological procedures do not record the yellow coloration during early stages of the reaction, and it is impossible to distinguish in the final black product between silver bound to phosphates and silver bound directly by other material.

Zill¹⁷ searched unsuccessfully for methods to prevent blackening of silver in von Kossa's² reaction. Apparently, this problem was not solved during the following decades. We, therefore, reinvestigated von Kossa's² and Zill's¹⁷ work.³

Histochemical Studies

In Vitro Experiments — Addition of silver nitrate solutions to reagent grade calcium carbonate and calcium phosphate yielded white silver carbonate and yellow silver phosphate. The former became deep gray when exposed to sunlight for 1.5 hours. In contrast, silver phosphate remained yellow during exposure to strong light on a sunny window sill for several days. Interestingly, silver carbonate prepared from the sodium (Na) salt became deep brown in dim light and black in strong illumination; i.e., von Kossa's reaction does not necessarily indicate calcium salts.

Because tissue sections are treated with sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) to remove unreacted silver, this reagent was added also in *in vitro* experiments. Silver phosphate was unaffected and remained yellow, but blackened silver carbonates dissolved in sodium thiosulfate. This observation is in agreement with chemical data by Hodgman et al.¹⁸ It seems probable that silver carbonates in calcium deposits are also subject to the laws of chemistry and dissolve in the sodium thiosulfate bath.

Upon addition of solutions of egg albumen or gelatin

to yellow silver phosphate crystals, the whole solution became dark brown to blackish, as described by von Kossa.² However, examination of smears of such material by polarization microscopy demonstrated virtually colorless crystals embedded in an amorphous brownish mass. Apparently, the protein solution did not reduce the silver phosphate to black silver, but available silver cations reacted with proteins, imparting a deep brown to black coloration. This process is analogous to familiar reactions when silver nitrate is spilled on skin or clothing and produces black discoloration without the benefit of calcium phosphate. According to Lillie¹⁹, the same reaction is given by soaps.

Studies of Tissue Sections — Because strong light is required for blackening of silver by organic material, but is not necessary for formation of yellow silver phosphate, it seemed sensible to perform von Kossa's² reaction in the absence of strong light. Four series of sections were placed in a 2% aqueous silver nitrate solution for one hour. Series I was exposed to sunlight as recommended by textbooks. Series II was kept in a shaded area of the laboratory. Series III was placed in a dark cabinet, and Series IV was wrapped in aluminum foil to exclude light. Sections were then rinsed in distilled water, placed in a 5% aqueous solution of sodium thiosulfate for five minutes, rinsed in distilled water, dehydrated, cleared and mounted as usual. In duplicate series, treatment with sodium thiosulfate was omitted.

Without exposure to sodium thiosulfate, sections from all four series contained black calcium deposits; i.e., reduction to metallic silver occurs also in the absence of light. These findings confirm observations by Pizzolato and McCrory.²⁰ After treatment with sodium thiosulfate, only sections of Series I, which had been exposed to sunlight, contained black material. In the other three series, the black coloration had vanished. Calcium deposits varied from yellowish brown to yellow to colorless; the latter could be identified by their refringence. There was no significant difference between Series II, III and IV. Thus, staining jars can be conveniently placed in a shady corner of the laboratory; special precautions to exclude light are not necessary. Treatment with sodium thiosulfate is essential to remove black material and to prevent gradual darkening of tissues. No darkening has been observed in sections stored for approximately 15 years.

The yellow to brownish-yellow color of many calcium deposits in Series II, III and IV corresponds to that of silver phosphate in *in vitro* experiments; other tissue structures remain unstained. Thus, the modified von Kossa's reaction can be regarded as a histochemical test for phosphates. However, the reaction seems to be limited to inorganic phosphates; nuclei and other sites known to contain nucleic acids are colorless, and it cannot visualize carbonates. As already mentioned, silver carbonate is soluble in sodium thiosulfate.¹⁸ Von Kossa's technique and its modifications are not reactions for calcium. Part of the phosphates in calcium deposits may be associated with cations other than calcium, therefore, reactions for calcium (for example, Alizarin Red S) and for phosphates may not always yield identical pictures, e.g., in arteriosclerotic lesions.²¹

Furthermore, chemical studies of calcification indicate that calcium is deposited first; phosphates appear some time later.²²

Conclusion

As emphasized already by von Kossa,² the black deposits formed in his reaction are due to reduction of silver by organic material. This artifact occurs only when sections are exposed to strong light as recommended in textbooks. When von Kossa's reaction is carried out in subdued light, yellow to yellowish-brown silver phosphate is visualized selectively. Silver carbonate cannot be demonstrated with von Kossa's technique because it dissolves in sodium thiosulfate solutions. Phosphates and carbonates in calcium deposits may be associated, at least in part, with cations other than calcium. Calcium can be identified with Alizarin Red S or other suitable mordant dyes.

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STUDY NUMBER 82
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GROSS NECROPSY OBSERVATIONS

IITRI PROJECT NUMBER LO6139

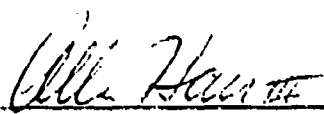
PHASE IV STUDY NO. 82

THE EFFECTS OF SUBCHRONIC EXPOSURES TO
RED PHOSPHORS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
ON VARIOUS BIOLOGICAL ENDPOINTS
IN MALE SPRAGUE-DAWLEY RATS

A summary of gross observations seen at necropsy in animals on this study follows:

Table 1 Terminal sacrifice animals
Table 2 Recovery sacrifice animals
Table 3 Spontaneous death animals

Examination of Tables 1, 2, and 3 fails to reveal a dose dependent compound induced lesion. All gross lesions seen are interpreted as incidental findings or agonal changes.


Allen Hall, III, D.V.M.,
Diplomate, ACVP

7/16/82
Date

GROSS SUMMARY TABLES

GROSS SUMMARY TABLES

TERMINAL SACRIFICES

Exposure Concentrations (mg/l)

215

ORGAN
Lest

GROSS SUMMARY TABLES

RECOVERY SACRIFICES

ORGAN Lesion	Exposure (mg/kg)	0.0	0.05	0.18	0.30
NUMBER OF RATS EXAMINED		20	20	20	20
NO GROSS LESIONS		18	18	15	18
LYMPH NODE(S)					
SUBMANDIBULAR					
Enlarged			2	1	
Red			2		
SALIVARY GLAND(S)					
Enlarged		1		1	1
Dark Red		1			
KIDNEY(S)					
Foci					1
LIVER					
Foci		1		1	
Mass				1	

Exposure
Concentrations (mg/l)

[illegible][illegible][illegible][illegible][illegible]

GROSS SUMMARY TABLES

RECOVERY SACRIFICES

Table 3

GROSS SUMMARY TABLES

SPONTANEOUS DEATHS

Exposure
Concentrations
(mg/l)Project L06139
Study Number 82
Test Article RP/BRORGAN
Lesion

0.30

0.18

0.05

0.0

NUMBER OF RATS EXAMINED				
NO GROSS LESION				
LYMPH NODE(S)				
SUBMANDIBULAR				
Dark red				
RESPIRATORY				
Enlarged, red				
THYMUS				
Enlarged				
LUNGS				
Pale				
Mottled				
Heart				
Enlarged, firm				

Table 3 (continued)
GROSS SUMMARY TABLES
SPONTANEOUS DEATHS

Exposure
Concentrations
(mg/l)

Project L06139
Study Number 82
Test Article RP/BR

ORGAN
Lesion

STOMACH	0.0	0.05	0.18	0.30
Bloated				1
INTESTINES				1
Dark red				
CECUM				1
Bloated				
SPLEEN			1	
Enlarged				1
Dark red				
LIVER				1
Granular, firm			1	
Enlarged, pale				
KIDNEY(S)			1	
Enlarged, pale				1
Granular, firm				

SPONTANEOUS DEATHS

Exposure Concentrations (mg/l)

Project L06139
Study Number 82
Test Article RP/BR

[illegible]

IITRI PROJECT NUMBER L06139
PHASE IV STUDY NUMBER 82
THE EFFECTS OF SUBCHRONIC EXPOSURES TO LOW CONCENTRATIONS OF
RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
ON HISTOPATHOLOGICAL CHANGES IN THE LUNGS AND ON
PULMONARY BACTERICIDAL ACTIVITY OF MALE SPRAGUE-DAWLEY RATS

PATHOLOGY REPORT

FINAL SACRIFICE, RECOVERY SACRIFICE, AND TWO MORTALITIES

Submitted To:

IIT Research Institute
10 West 35th Street
Chicago, IL 60616

Submitted By:

Experimental Pathology Laboratories, Inc.
Midwest Laboratory
1800 E. Pershing Road
Decatur, IL 62526

May 5, 1986

QUALITY ASSURANCE
REPORT CERTIFICATION

Client Name: IIT Research Institute

Client Study Number: L06139, Phase IV, Study Number 82

Study Coordinator: Dr. S.V. Becker

Pathologist: Dr. W.O. Iverson

Study Title: The Effects of Subchronic Exposures to Red Phosphorus/Butyl Rubber (RP/BR) Combustion Products on Histopathological Changes in the Lungs and on Pulmonary Bactericidal Activity of Male Sprague-Dawley Rats.

Test Article: Combustion Products of Red Phosphorus/Butyl Rubber

Species: Sprague-Dawley Rat

All parts of the pathology phase of this study, including the final report, were reviewed by Experimental Pathology Laboratories Quality Assurance Unit on January 12, February 8, March 14, and April 22 and 24, 1986. All findings were reported to the Study Coordinator and Management.

Susan M. Hout
Susan M. Hout

5/7/86

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PATHOLOGY SUMMARY

IITRI PROJECT NUMBER L06139
 PHASE IV STUDY NUMBER 82
 THE EFFECTS OF SUBCHRONIC EXPOSURES TO LOW CONCENTRATIONS OF
 RED PHOSPHORUS/BUTYL RUBBER (RP/BR) COMBUSTION PRODUCTS
 ON HISTOPATHOLOGICAL CHANGES IN THE LUNGS AND ON
 PULMONARY BACTERICIDAL ACTIVITY OF MALE SPRAGUE-DAWLEY RATS

FINAL SACRIFICE, RECOVERY SACRIFICE, AND TWO MORTALITIES

PATHOLOGY SUMMARY

Microscopic examinations were performed on selected tissues from male Sprague-Dawley rats. The purpose of this study was to evaluate the effects of exposure concentration and recovery time of the repeated exposure of rats to combustion products of Red Phosphorus/Butyl Rubber (RP/BR) on histopathological changes in the lungs and on pulmonary bactericidal activity. This report contains the histopathologic findings for the terminal sacrifices, recovery sacrifices, and two animals which died spontaneously. The experimental design for this study was as follows:

T.G. No.	Freq/ Start Code	Endpt. Group	Expo. Freq.	Expo. Conc.	Recov.	Exposure Start	Exposure End	Exper. Date	Animal Numbers
17	I	PATH	F2	C0	-	8/12	11/7	11/8	337-346
18	I	PATH	F2	C1	-	8/12	11/7	11/8	347-356
19	I	PATH	F2	C2	-	8/12	11/7	11/8	357-366
20	I	PATH	F2	C3	-	8/12	11/7	11/8	367-376
21	II	PATH	F2	C0	-	8/19	11/14	11/15	377-386
22	II	PATH	F2	C1	-	8/19	11/14	11/15	387-396
23	II	PATH	F2	C2	-	8/19	11/14	11/15	397-406
24	II	PATH	F2	C3	-	8/19	11/14	11/15	407-416
25	I-R	PATH	F2	C0	+	8/12	11/7	1/2	417-426
26	I-R	PATH	F2	C1	+	8/12	11/7	1/2	427-436
27	I-R	PATH	F2	C2	+	8/12	11/7	1/2	437-446
28	I-R	PATH	F2	C3	+	8/12	11/7	1/2	447-456
29	II-R	PATH	F2	C0	+	8/19	11/14	1/9	457-466
30	II-R	PATH	F2	C1	+	8/19	11/14	1/9	467-476
31	II-R	PATH	F2	C2	+	8/19	11/14	1/9	477-486
32	II-R	PATH	F2	C3	+	8/19	11/14	1/9	487-496

T.G. NO. = Treatment group number

All animals were exposed for 2.25 hr/day on four consecutive days per week.

Doses administered were as shown below:

C0 = Filtered air control
C1 = 0.05 mg/l
C2 = 0.18 mg/l
C3 = 0.30 mg/l

The animals sacrificed on 11/8/85 and 11/15/85 were designated the final sacrifice. Animals sacrificed on 1/2/86 and 1/9/86 were designated the recovery sacrifice. Animals 323 and 311 were not part of the designated evaluation group but did die and are reported here.

Complete necropsies of all rats designated for pathology were conducted. The following tissues were processed to paraffin blocks for all animals which died or were sacrificed and designated for pathology: one level of nasal turbinate, trachea, pulmonary lymph node, lung and gross lesions. All five lobes of the lung were processed and examined. All paraffin blocks were then shipped to Experimental Pathology Laboratories, Inc. where hematoxylin and eosin stained slides of these tissues were prepared and examined for all even animal numbers. All odd animal numbers had all blocks except the nasal turbinate and trachea sectioned and examined. Additional lung sections from all animals were stained with Masson's trichrome stain for demonstration of collagen.

RESULTS

The microscopic changes and a detailed listing of all tissues evaluated are presented in the Histopathology Incidence Tables. All lesions are summarized by treatment group and presented in the Summary Incidence Tables. A correlation of lesions observed at necropsy with the corresponding microscopic observation, where possible, is presented in the Correlation of Gross and Microscopic Findings Tables. The gross observations in these tables were transcribed from the necropsy sheets

provided with the paraffin blocks. Animal dispositions were listed as final sacrifice or spontaneous death as they were indicated on the necropsy sheets.

The primary treatment-related change seen histologically in this study was in the lung and was diagnosed as "terminal bronchiolar fibrosis". The lesion consisted of minimal thickening of the alveolar walls and of the most distal portions of the terminal bronchioles at the point where the terminal bronchiole ends and joins the alveolar sacs. The thickening consisted of a heterogenous eosinophilic material containing small numbers of cells. This material stained positively for collagen with Masson's trichrome stain. The incidence of this lesion is shown in the following table:

INCIDENCE OF TERMINAL BRONCHIOLAR FIBROSIS

Severity	Group:	Number of Animals							
		Final Sacrifice				Recovery Sacrifice			
		C0	C1	C2	C3	C0	C1	C2	C3
Minimal -- Grade 1		0/20	0/20	4/20	9/20	0/20	0/20	3/20	4/20

Other changes in the lung were usually minimal or absent altogether. Some animals had small numbers of alveolar macrophages but they were usually not associated with the terminal bronchiolar fibrosis. Minimal to mild lymphocytic hyperplasia occurred in many of the animals, both treated and controls. Other changes were relatively infrequent.

Two animals which died spontaneously were submitted for examination. Animal 323 had chronic hemorrhage and congestion of the lung. Animal 311 had a histocytic sarcoma on one leg, which was probably contributory to its death.

CONCLUSION

The results of these microscopic examinations indicate that the exposure of male Sprague-Dawley rats to the combustion products of RP/BR for 2.25 hr/day for four consecutive days per week produced minimal terminal bronchiolar fibrosis in less than 50% of the animals after three months of exposure at 0.30 mg/l and less than 25% of the animals after three months of exposure at 0.18 mg/l. The lowest dose, 0.05 mg/l, appears to be a no-effect level. Following an eight-week recovery period there is a decreased incidence of the lesion, but it still is present. There were no other changes found in the tissues examined that were treatment-related.

W.O. Iverson, D.V.M.
W.O. Iverson, D.V.M.
Diplomate, ACVP

May 5, 1986

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

LO6139 PHASE IV

STUDY NUMBER 82

FINAL SACRIFICE

GROUP:	C0	C1	C2	C3		
NASAL TURBINATE						
(Number Examined)	(10)	(10)	(10)	(10)		
Hemorrhage	0	0	1	1		
Eosinophilic Globules	0	0	0	0		
TRACHEA						
(Number Examined)	(10)	(10)	(10)	(10)		
PULMONARY LYMPH NODE						
(Number Examined)	(20)	(20)	(20)	(20)		
Hemorrhage	13	7	7	11		
Lymphocytic Hyperplasia	4	2	2	1		
Pigment	4	4	7	3		
Edema	0	4	4	1		
LUNG						
(Number Examined)	(20)	(20)	(20)	(20)		
Atelectasis	6	7	7	5		
Hemorrhage	4	7	7	8		
Focal Lymphocytic Aggregate	2	3	3	3		
Alveolar Macrophages	1	0	3	2		
Interstitial Inflammation	0	1	0	0		
Terminal Bronchiolar Fibrosis	0	0	4	9		
Eosinophilic Infiltrate	0	0	0	0		
Mineralization	0	0	0	0		

SUMMARY INCIDENCE TABLE

L06139 PHASE IV

STUDY NUMBER 82

FINAL SACRIFICE

GROUP:	C0	C1	C2	C3		
KIDNEY						
(Number Examined)	(3)	(2)	(1)	(2)		
Hyaline Casts	2	2	1	2		
Tubular Hyperplasia	2	0	0	1		
Cyst	0	0	0	1		
TESTIS						
(Number Examined)	(0)	(1)	(0)	(0)		
Hemorrhage	0	1	0	0		
SALIVARY LYMPH NODE						
(Number Examined)	(1)	(0)	(0)	(0)		
Hemorrhage	1	0	0	0		
Lymphocytic Hyperplasia	1	0	0	0		
Pigment	0	0	0	0		
LIVER						
(Number Examined)	(0)	(0)	(0)	(0)		
Hyperplasia	0	0	0	0		
HEART						
(Number Examined)	(0)	(0)	(0)	(0)		
LACRIMAL GLAND						
(Number Examined)	(0)	(0)	(0)	(0)		
Inflammation	0	0	0	0		
PREPUTIAL GLAND						
(Number Examined)	(0)	(0)	(0)	(0)		
Abscess	0	0	0	0		

E P L

SUMMARY INCIDENCE TABLE

L06139 PHASE IV

STUDY NUMBER 82

FINAL SACRIFICE

[illegible]

SUMMARY INCIDENCE TABLE

L06139 PHASE IV

STUDY NUMBER 82

RECOVERY SACRIFICE

GROUP:	C0	C1	C2	C3		
NASAL TURBINATE						
(Number Examined)	(10)	(10)	(10)	(10)		
Hemorrhage	0	1	1	0		
Eosinophilic Globules	1	2	3	4		
TRACHEA						
(Number Examined)	(10)	(10)	(10)	(10)		
PULMONARY LYMPH NODE						
(Number Examined)	(20)	(20)	(20)	(20)		
Hemorrhage	1	3	6	3		
Lymphocytic Hyperplasia	15	10	11	13		
Pigment	3	2	3	6		
Edema	3	4	2	0		
LUNG						
(Number Examined)	(20)	(20)	(20)	(20)		
Atelectasis	7	10	5	7		
Hemorrhage	4	4	3	6		
Focal Lymphocytic Aggregate	5	3	0	4		
Alveolar Macrophages	2	3	4	5		
Interstitial Inflammation	2	0	0	2		
Terminal Bronchiolar Fibrosis	0	0	3	4		
Eosinophilic Infiltrate	3	0	0	0		
Mineralization	0	3	2	2		

SUMMARY INCIDENCE TABLE

L06139 PHASE IV

STUDY NUMBER 82

RECOVERY SACRIFICE

GROUP:	C0	C1	C2	C3		
KIDNEY						
(Number Examined)	(0)	(0)	(0)	(1)		
Hyaline Casts	0	0	0	1		
Tubular Hyperplasia	0	0	0	1		
Cyst	0	0	0	0		
TESTIS						
(Number Examined)	(0)	(0)	(0)	(0)		
Hemorrhage	0	0	0	0		
SALIVARY LYMPH NODE						
(Number Examined)	(1)	(2)	(1)	(0)		
Hemorrhage	1	2	1	0		
Lymphocytic Hyperplasia	1	2	1	0		
Pigment	0	1	0	0		
LIVER						
(Number Examined)	(1)	(0)	(1)	(0)		
Hyperplasia	0	0	1	0		
HEART						
(Number Examined)	(0)	(0)	(1)	(0)		
LACRIMAL GLAND						
(Number Examined)	(0)	(0)	(1)	(0)		
Inflammation	0	0	1	0		
PREPUTIAL GLAND						
(Number Examined)	(0)	(0)	(0)	(1)		
Abscess	0	0	0	1		

EPL

SUMMARY INCIDENCE TABLE

L06139 PHASE IV

STUDY NUMBER 82

RECOVERY SACRIFICE

[illegible]

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

L A B O R A T O R Y N U M B E R	G R O U P	C O																					
			337	338	339	340	341	342	343	344	345	346	377	378	379	380	381	382	383	384	385	386	
06139 PHASE IV																							
STUDY NUMBER 82																							
FINAL SACRIFICE																							
GROUP C0																							
NASAL TURBINATE																							
TRACHEA																							
PULMONARY LYMPH NODE																							
Hemorrhage																							
Lymphocytic Hyperplasia																							
Pigment																							
LUNG																							
Atelectasis																							
Hemorrhage																							
Focal Lymphocyte Aggregate																							
Alveolar Macrophages																							
KIDNEY																							
Hyaline Casts																							
Tubular Hyperplasia																							

11

Key P = Present 1 = Minimal
 N = No Section 2 = Slight 3 = Moderate 4 = Not Remarkable
 1 = Incomplete Section

EPL Experimental Pathology Laboratories, Inc.

HISTOPATHOLOGY INCIDENCE TABLE

106139 PHASE IV	GROUP CO	SALIVARY LYMPH NODE	Hemorrhage	Lymphocytic Hyperplasia	1	2	337	338	339	340	341	342	343	344	345	346	377	378	379	380	381	382	383	384	385	386																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
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HISTOPATHOLOGY INCIDENCE TABLE

L06139 PHASE IV STUDY NUMBER 02 FINAL SACRIFICE GROUP C1	NUMBER ANIMALS																				
		347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366
MASAL TURBIDATE																					
TRACHEA																					
PULMONARY LYMPH NODE																					
Hemorrhage																					
Lymphocytic Hyperplasia																					
Pigment																					
Edema																					
LUNG																					
Atelectasis																					
Hemorrhage																					
Focal Lymphocyte Aggregate																					
Interstitial Inflammation																					
KIDNEY																					
Hyaline Casts																					

NUMBER
ANIMALS

L06139 PHASE IV
STUDY NUMBER 82
FINAL SACRIFICE
GROUP C1

11 12 13 14 15
 A N : M A :

TESTIS
Hemorrhage

[illegible]

此

Key P = Present
I = Minimal

$N = \text{No. of } \text{CH}_2$
 $2 = \text{Steps}$

A = Antibody
B = Molecule

Dr. - Not known, etc.

HISTOPATHOLOGY INCIDENCE TABLE

Z D M B L R
X Z - M A L

L06139 PHASE IV

STUDY NUMBER 82

FINAL SACRIFICE

GROUP C2

[illegible]

15

193

Key: P = Present
1 = Manual

2 = No Section
3 = Start

$\lambda =$ Autolysis
 $\lambda =$ Moderate

X - Not Remarkable
J - Moderately Sincere High

Experimental Pathology Laboratories, Inc.

LO6139 PHASE IV
 STUDY NUMBER 82
 FINAL SACRIFICE
 GROUP C2

ZIMMER
ANN-MAL

KIDNEY
Hyaline Casts

406	
405	
404	
403	
402	
401	
400	
399	
398	
397	
396	
395	
394	
393	
392	
391	
390	-
389	
388	
387	

16

183

Key **P** = Present
 I = Minimal

2. No Section

A = Autolytic
M = Moderate

X = Not remarkable
A = Moderately severe flap

Department of Psychology Laboratories, Inc.

HISTOPATHOLOGY INCIDENCE TABLE

L06139 PHASE IV STUDY NUMBER 82 FINAL SACRIFICE GROUP C3		NUMBER ANIMAL																
NASAL TURBINATE Hemorrhage																		
TRACHEA																		
PULMONARY LYMPH NODE Hemorrhage																		
Lymphocytic Hyperplasia																		
Pigment																		
Edema																		
LUNG																		
Atelectasis																		
Hemorrhage																		
Focal Lymphocyte Aggregate																		
Alveolar Macrophages																		
Terminal Bronchiolar Fibrosis																		

NUMBER
ANIMAL

L06139 PHASE IV
STUDY NUMBER 82
FINAL SACRIFICE
GROUP C3

NASAL TURBINATE
Hemorrhage

TRACHEA

PULMONARY LYMPH NODE
Hemorrhage

Lymphocytic Hyperplasia

Pigment

Edema

LUNG

Atelectasis

Hemorrhage

Focal Lymphocyte Aggregate

Alveolar Macrophages

Terminal Bronchiolar Fibrosis

EPL

17

Key P = Present
1 = Minimal

N = No Reaction
2 = Slight

A = Antidysplastic
3 = Moderate
4 = Not Remarkable

Experimental Pathology Laboratories, Inc.

L06139 PHASE IV
STUDY NUMBER 82
FINAL SACRIFICE
GROUP C3

Z - M R - R
A Z - M A L

X = Not Removable

HISTOPATHOLOGY INCIDENCE TABLE

LO6139 PHASE IV
STUDY NUMBER 82
RECOVERY SACRIFICE
GROUP C0

NUMBER
ANIMAL

	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446
NASAL TURBINATE	X		X	X		X		X		X		X		X		X		X		X		X		X		X		X		X
Eosinophilic Globules																														
TRACHEA	X	X		X		X		X		X		X		X		X		X		X		X		X		X		X		X
PULMONARY LYMPH NODE					X																									
Hemorrhage									1																					
Lymphocytic Hyperplasia	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pigment	1								1																					
Edema			1								2																			
LUNG																														
Atelectasis																														
Hemorrhage																														
Focal Lymphocytic Aggregate	1																													
Alveolar Macrophages			1																											
Interstitial Inflammation																														
Eosinophilic Infiltrate																														

X = Not Remarkable
1 = Moderately Severe
2 = Moderately Severe
3 = Severe
4 = Very Severe

A = Autolysis
3 = Moderate
4 = Severe

N = No Section
2 = Slight
1 = the complete Section

Key P = Present
1 = Minimal

19

Z - M B F R
A Z - M A L

SALIVARY LYMPH NODE

Hemorrhage

Lymphocytic Hyperplasia

LIVER

[illegible]

193

Key P = Present

20

N = No Section

A = Autolysis

X = Not Remarkable

HISTOPATHOLOGY INCIDENCE TABLE

L06139 PHASE IV
STUDY NUMBER 82
RECOVERY SACRIFICE
GROUP C1

	427	428	429	430	431	432	433	434	435	436	467	468	469	470	471	472	473	474	475	476
NASAL TURBINATE		X				X				X	X	X	X	X		X		X		
Hemorrhage																				2
Eosinophilic Globules			1					1												
TRACHEA	X	X		X		X		X		X		X		X		X		X	X	
PULMONARY LYMPH NODE				X			X	X	X							X				
Hemorrhage	1	1								1							1			
Lymphocytic Hyperplasia			1							2	1	2	2	1	1	1	1	1	2	1
Pigment		1																	1	
Edema					1	1								1					1	
LUNG											X		X						X	X
Atelectasis	1	1	1	1			1		1			2		2	1	2	2			
Hemorrhage	1	1	1												1	1				
Focal Lymphocytic Aggregate						1					1	1								
Alveolar Macrophages						1		1								1				
Mineralization				1										1	1					

21

Key P = Present
1 = Minimal

N = No Section
2 = Cough

A = Autolysis
3 = Moderate

X = Not Remarkable
3 = Moderate

EPL Experimental Pathology Laboratories, Inc.

Z D M B F B
A Z - M A -

SALIVARY LYMPH NODE
Hemorrhage
Lymphocytic Hyperplasia
Pigment

[illegible]

Key: P = Present
1 = Minimal
2 = Slight
3 = Moderate
4 = Moderately Severe
X = Not Remarkable

HISTOPATHOLOGY INCIDENCE TABLE

NUMBER
A 2 - MAY

LO6132 PHASE IV
STUDY NUMBER 82
RECOVERY SACRIFICE
GROUP C2

L06130 PHASE IV STUDY NUMBER 82 RECOVERY SACRIFICE GROUP C2		ANUMBER	
NASAL TURBINATE		437	438
Hemorrhage			
Eosinophilic Globules			
TRACHEA			
PULMONARY LYMPH NODE			
Hemorrhage			
Lymphocytic Hyperplasia			
Pigment			
Edema			
LUNG			
Atelectasis			
Hemorrhage			
Alveolar Macrophages			
Terminal Bronchiolar Fibrosis			
Mineralization			

X = Not Remarkable
4 = Moderately Severe High

A = Autolysis
3 = Moderate

1 = No Section
2 = Slight
3 = Incomplete Section

Key: P = Present

Experimental Pathology Laboratories, Inc.

L06139 PHASE IV
STUDY NUMBER 82
RECOVERY SACRIFICE
GROUP C2

N = No Section
2 = Slight
I = Incomplete Section

A = Autolysis
3 = Moderate
X = Not Remarkable

4 = Moderately Severe
High

HISTOPATHOLOGY INCIDENCE TABLE

NUMBER ANIMAL		447	448	449	450	451	452	453	454	455	456	487	488	489	490	491	492	493	494	495	496
L06139 PHASE IV																					
STUDY NUMBER 82																					
RECOVERY SACRIFICE																					
GROUP C3																					
NASAL TURBINATE																					
Eosinophilic Globules		1									1	2									1
TRACHEA																					
PULMONARY LYMPH NODE																					
Hemorrhage																					
Lymphocytic Hyperplasia																					
Pigment																					
LUNG																					
Atelectasis																					
Hemorrhage																					
Focal Lymphocytic Aggregate																					
Alveolar Macrophages																					
Interstitial Inflammation																					
Terminal Bronchiolar Fibrosis																					
Mineralization																					

Key P = Present 1 = Minimal
 N = No Section 2 = Moderate
 A = Autolysis 3 = Marked
 X = Not Remarkable

NUMBER
AN-MAL

KIDNEY
Hyaline Casts
Tubular Hyperplasia

Abscess

SALIVARY GLAND

26

Key P = Present
1 = Minimal

N = No Section
2 = Supply

A = Analysis
3 = Moderate

$X = \text{unweighted average}$
 $A = \text{weighted average}$

Experimental Pathology Laboratories, Inc.

IITRI PROJECT NUMBER L06139
PHASE IV STUDY NUMBER 82

SPONTANEOUS DEATHS
HISTOPATHOLOGY REPORT

<u>TISSUE</u>	<u>FINDING</u>
ANIMAL NUMBER 323	
LUNG	Congestion, Moderate Pigment, Mild Atelectasis, Mild Alveolar Macrophages, Mild
PULMONARY LYMPH NODE	Hemorrhage, Moderate Pigment, Moderate
HEART	Normal
SPLEEN	Pigment, Mild
PANCREAS	Hemorrhage, Moderate
SALIVARY LYMPH NODE	Congestion
KIDNEY	Hyaline Casts, Mild
THYMUS	Lymphoid Depletion, Moderate Pigment, Moderate Hemorrhage, Mild
TESTIS	Normal
PROSTATE	Normal
SEMINAL VESICLE	Normal
STOMACH	Normal
DUODENUM	Congestion, Minimal
JEJUNUM	Autolysis
ILEUM	Autolysis
CECUM	Autolysis
COLON	Autolysis
ADRENAL	Congestion, Mild
ANIMAL NUMBER 311	
LUNG	Congestion, Minimal
PULMONARY LYMPH NODE	Lymphocytic Hyperplasia, Minimal
LIVER	Normal
HEART	Normal
SPLEEN	Congestion, Minimal
KIDNEY	Tubular Hyperplasia, Minimal Hyaline Casts, Minimal
TISSUE MASS	Histiocytic Sarcoma

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS TABLES

STUDY NUMBER 82

FINAL SACRIFICE

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS

[illegible]

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS

0.05 mg/l

Dosage Level

Group Number: C1

Sex Males

[illegible]

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS

Sex: Males

Group Number: C2

Dosage Level	0.13 mg/l
--------------	-----------

[illegible]

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS

Dosage Level 0.30 mg/l

Group Number: C3

Sex: Males

[illegible]

RECOVERY SACRIFICE

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS

Dosage Level: Filtered air control

[illegible]

RECOVERY SACRIFICE

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS

Dosage Level	0.05 mg/l
--------------	-----------

C1

Group Number:

Sex: Males

Species: Rats

[illegible]

L06139 PHASE IV

STUDY NUMBER 82

RECOVERY SACRIFICE

CORRELATION OF GROSS AND MICROSCOPIC FINDINGS

Dosage Level: 0.18 mg/l

Group Number: C2

Sex: Males

Species: Rats

Animal Number	Client's Tissue Identification	Client's Gross Observations	Microscopic Observations
441	SALIVARY GLAND	Right, enlarged in size	No Corollary Change Detected
443	LIVER	Dark red focus on diaphragmatic surface of left lateral lobe, 0.1-0.2 cm	No Corollary Change Detected
444	LIVER	Mass involving median lobe of liver, 33 mm x 15 mm	Hyperplasia
446	HEART	Small depressed area at apex	No Corollary Change Detected
	EYE	Eyes, dark discoloration of lacrimal glands	Inflammation
434	SALIVARY LYMPHS	Enlarged	Lymphocytic Hyperplasia

RECOVERY SACRIFICE

Dosage level 0.30 mg/l

[illegible]

APPENDIX B

IN VITRO GENETIC TOXICOLOGY TESTING OF RP/BR AEROSOL CONDENSATE

FINAL REPORTS

Prepared by

ROBERT R. GUERRERO

**AMES SALMONELLA TYPHIMURIUM REVERSE MUTATION ANALYSIS OF AN EXTRACT
OF RED PHOSPHORUS/BUTYL RUBBER AEROSOL CONDENSATE**

**CHROMOSOME ABERRATION ANALYSIS OF AN EXTRACT OF RED PHOSPHORUS/BUTYL
RUBBER AEROSOL CONDENSATE**

**DNA REPAIR ASSAY IN PRIMARY RAT HEPATOCYTES ON AN EXTRACT OF RED
PHOSPHORUS/BUTYL RUBBER AEROSOL CONDENSATE**

FORWORD

These final reports on studies entitled "Ames Salmonella typhimurium Reverse Mutation Analysis of an Extract of Red Phosphorus/Butyl Rubber Aerosol Condensate", "Chromosome Aberration Analysis of an Extract of Red Phosphorus/Butyl Rubber Aerosol Condensate", and DNA Repair Assay in Primary Rat Hepatocytes on an Extract of Red Phosphorus/Butyl Rubber Aerosol Condensate" were prepared by Robert Guerrero, Genetic Toxicologist. Methodology and personnel needed to generate the RP/BR aerosol from which the "RP/BR Condensate Test Article" for the genetic toxicology tests was produced were provided through the main study. The work was supported with additional funds provided under the main contract (No. DAMD17-82-C-2121) specifically for these studies.

Catherine Aranyi

Catherine Aranyi
Scientific Advisor
Inhalation Toxicologist
Principal Investigator

FINAL REPORT

AMES SALMONELLA TYPHIMURIUM REVERSE MUTATION ANALYSIS OF AN EXTRACT
OF RED PHOSPHORUS/BUTYL RUBBER AEROSOL CONDENSATE

IITRI Project No. LO6139L001
Study No. 81A

May 1985

IIT RESEARCH INSTITUTE

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1. INTRODUCTION

The purpose of this study was to assess the mutagenic properties of an extract of red phosphorus/butyl rubber condensate and to determine the influence of pH on the mutagenic response. The study covers protocols No. SN81A USA-01 and USA-02.

Red phosphorus butyl rubber (RP/BR) aerosol condensate is about 95% phosphoric acid. Therefore, addition of even small amounts of the agent to the bacterial cultures constitutes a severe pH challenge to the organisms.

In a recent workshop on the effects of low pH and high salt concentrations on genotoxicity *in vitro* (EMS workshop, Las Vegas, Nevada, February 1985), it was reported that low pH and high osmolality conditions can induce false positive results in *in vitro* systems. Therefore, a preliminary round of testing was done with phosphoric acid alone to determine the pH range the *S. typhimurium* bacteria would tolerate and to determine whether low pH alone would induce false positive results and finally whether neutralizing the acid to pH 7.0 with sodium hydroxide would cause a false positive response due to the increased osmolality. Subsequently the RP/BR test article was tested at pH 1.9, 6.0 and 7.0 with phosphoric acid pH controls for the three doses. The USA-01 (RP/BR) sample and the H_3PO_4 controls were diluted with distilled water (pH 6.2) to achieve pH 6 rather than use of NaOH as in the dose range finding study. However, since the water's pH was 6.2, a nominal amount of NaOH was used to raise the pH from 6 to 7 for the pH 7 dose. Use of H_2O instead of NaOH to adjust pH tested the osmolality variable. This study design enabled us to control all the known variables yet still assay the test article (USA-01) at the maximal dose.

The Ames test is an *in vitro* test that detects mutagens by their ability to cause base-pair and frameshift mutations. Five mutant auxotrophic strains of *Salmonella typhimurium*, deficient in the enzymes necessary to synthesize histidine, are used to measure DNA damage. The test measures point mutations at the histidine locus which revert the strains back to the prototrophic forms. As such, they regain ability to synthesize histidine and can grow in histidine deficient medium. Therefore, a test article's mutagenic potential can be determined by the number of revertants that form following exposure to it. When coupled with a rat liver microsomal fraction (S-9), the system simulates *in vivo* metabolic activation and detoxification pathways. As such, the assay provides a sensitive, fast and relatively inexpensive system for screening mutagens.

2. DATA ANALYSIS

A test was considered positive if the number of revertants was $\geq 2X$ the spontaneous background level.

3. TEST ARTICLE AND CONTROL INFORMATION

a. Test Article (USA-Q1): RP/BR was generated by burning RP/BR in a hydraulic driven extrusion-combustion generator on February 12 and 13, 1985. The extrusion/burning rate was adjusted to produce a final concentration of approximately 1 mg/l inside a 1 m³ stainless steel inhalation chamber. The aerosol was collected by condensation in liquid oxygen cooled Dewar traps from 9×10^3 liters of aerosol. The sampling rate was approximately 10 liters/min for a residence time in the traps of 30 seconds. The traps were opened and the inner walls were rinsed with 30 ml of methylene chloride (MC). The traps were drained into a 1 liter glass separatory funnel and the MC and the aqueous fractions were separated and transferred to 200 ml graduated cylinders. This procedure resulted in approximately 150 ml MC fraction and 105 ml aqueous fraction. The MC was driven off to dryness with a stream of dry nitrogen. The resultant yellow residue was dissolved in 2 ml DMSO.

The aqueous phase was transferred to a 150 ml round bottom flask. The aqueous sample was concentrated over a 2-day period to 7 ml with vacuum pressure delivered by a mechanical vacuum pump equipped with a liquid nitrogen cold trap. A 0.0304 g sample of the viscous yellowish-brown liquid was diluted to 100 ml with distilled water and three aliquots were analyzed for phosphate content. The resultant phosphoric acid concentration was determined to be 90.6% by weight.

For genotoxicity testing, the remaining aqueous fraction (5.64g) was combined with the 2 ml of DMSO MC extract and the mixture was diluted to 14 ml with ASTM type 1 water on February 19, 1985. The dilution was made to provide enough sample for three *in vitro* assays. The test article was stored at room temperature protected from light in a sterile pyrex glass tube. The density of the test article was 1.16 g/ml.

b. Control Substances:

Non-Activated System (-S-9)

Strain	Compound	Source	Solvent	Conc. (ug/plate)
TA98	2-Nitrofluorene (2NF)	Aldrich N1, 675-4	DMSO	10
TA100	Sodium Azide (NAZ)	Tridom 71290	H ₂ O	10
TA1535	Sodium Azide (NAZ)	Tridom 71290	H ₂ O	10
TA1537	9-Aminocridine (9A)	SIGMA A0510	Ethanol	100
TA1538	2-Nitrofluorene (2NF)	Aldrich N1, 675-4	DMSO	10

Activated System (+S-9)

Strain	Compound	Source	Solvent	Conc. (ug/plate)
All	2-Anthramine (2A)	Aldrich A3,880-0	DMSO	10

Phosphoric Acid pH Controls

Strain	pH	Acid Source	Solvent	Conc. (ul/plate)
All	1.87	Baker 1-0260	Distilled water	100
All	5.90	Baker 1-0260	Distilled water	100
All	6.95	Baker 1-0260	Distilled water	100

Distilled Water Control

Strain	pH	Source	Conc. (ul/plate)
All	6.9	DIFCO	100

c. Salmonella Tester Strains

The Salmonella typhimurium strains used in this study were TA98, TA100, TA1535, TA1537 and TA1538. The strains were obtained from Dr. Bruce Ames in October 1975. All strains are histidine deficient variants of the prototrophic wild type. Strains TA1537, TA1538 and TA98 detect frame shift reverse mutations at the histidine locus and TA1535 and TA100 detect base-pair substitution reverse mutations at the same locus. Stock cultures were grown and checked to confirm their mutational characteristics on September 12, 1980. The stocks were stored frozen at -70°C in nutrient broth/DMSO.

1) Strain Characteristics

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		LPS	Repair	B Factor	
TA98	his D	rfa	uvr B	pKM101	Frameshift
TA100	his G	rfa	uvr B	pKM101	Base-pair substitution
TA1535	his G	rfa	uvr B	--	Base-pair substitution
TA1537	his C	rfa	uvr B	--	Frameshift
TA1538	his D	rfa	uvr B	--	Frameshift

2) Spontaneous Reversion Rates

Occurrence of spontaneous revertants was used to assess tester strain response. For the test to be considered valid, the number of spontaneous revertants had to fall within the range characteristic for each strain.

Spontaneous Revertant Standard Count without S-2

Strain	Allowable Revertants
TA98	20-50
TA100	120-200
TA1535	10-35
TA1537	3-15
TA1538	15-35

4. METHODS

a. Experimental Design: The test article was tested for toxicity against *Salmonella* tester strain, TA98, both with and without S-9 prior to the mutagenicity test. The results of the toxicity test were used to establish the dosage range for the mutagenicity assay. For the mutagenicity test, the test article and 10^8 colony forming units (cfu) tester bacteria were combined and poured over minimal agar plates and after incubation at 37°C for 2 days histidine revertants were scored. Appropriate solvent and positive mutagens served as controls. Mutagenicity tests were performed using triplicate plates for each tester strain, with and without S-9, at each dose level. Sterility tests were performed on each test dose, as well as on the minimal medium, S-9 product, S-9 mix and overlay agar. The sterility tests were done on the same day the test was performed and under the same incubation and time conditions.

b. Dosage Formulations: For the toxicity test a 100 mg/ml stock solution was made by diluting 0.216 ml (250 mg) of the test article to 2.50 ml with distilled water. For the mutagenicity test a 50 mg/ml stock solution was made by diluting 0.432 ml (500 mg) of the test article to 10 ml with distilled water.

The dose levels tested for toxicity were 5.0 mg, 2.5 mg, 1.25 mg, 0.50 mg, 0.05 mg and 0.005 mg per plate.

The dose levels tested for mutagenicity were 2.5 mg (pH 1.90), 0.119 ug (pH 6.0) and 0.119 ug (adjusted to pH 7.0 with 0.1 N NaOH) per plate.

c. Chemical Handling and Preparation: Upon receipt of the test and control articles, the date of receipt and quantities were recorded and a reference sample was taken. Liquid test articles were measured by weight. All test and control substances were freshly prepared the day of use and were blended by vortex mixing for 1 minute. The control compounds were stored according to manufacturers' recommendations regarding temperature, humidity and protection from light. Dilutions of test and control compounds were prepared in distilled water, ethanol (US1) or Spectr-AR™ grade (Mallinckrodt) dimethylsulfoxide (DMSO) and were used in the plate assay within 1 hr of preparation. Unused pyrex glass tubes were used for preparing all dilutions.

d. Preparation of Liver Homogenates and Reaction Mixture: A 9,000 xg microsomal supernate isolated from livers of adult male Fischer 344 rats induced with Aroclor 1254 was used to activate promutagens. The S-9 was prepared as per Ames et al. (Mutation Research 31:347-364, 1975) and was purchased from Litton Bionetics, Kensington, MD (Lot No. 04142). Upon receipt the S-9 was stored at -70°C until used. Lot 04142 contained 21.5 mg/ml protein and had p448/p450 activity equivalent to 5.9 nMol/mg protein. Benzo(α)pyrene activated by this S-9 induced 757 and 814 revertants in strains TA98 and TA100 respectively. The reacting mixture was prepared just prior to use. The S-9 mix was sterilized by filtration through a 0.45 μm membrane filter then the liver fraction was added just prior to use. The S-9 reaction mixture was ultimately comprised of 450 ul of the S-9 mix and 50 ul of rat liver homogenate.

e. Preparation of Minimal Agar Plates and Top Agar: The minimal medium plates used throughout the study contained 20 ml of Vogel-Bonner medium E (Vogel and Bonner, J. Biol. Chem. 218:97-106, 1956) with 2% dextrose. Top agar was autoclaved, then L-histidine-HCL and biotin were added just prior to use. The ingredients were mixed then 2 ml were added to unused 16x150 mm disposable glass test tubes and allowed to equilibrate to 45°C in a waterbath before addition of any bacteria or chemicals.

f. Dose range finding study: A spot test dose range finding study was performed using strain TA98 only. The assay was a modification of the standard disk susceptibility assay as described by Bauer et al. in Amer. J. Clin. Path. 45:493-496, 1966. Briefly, a 24 hr culture of TA98 was inoculated onto Oxoid nutrient agar plates. The test article was applied to sterile filter paper disks (S and S, 740-E) in a dose related manner up to 5 mg/disk. The disks were transferred to the inoculated plates and after a 15 minute incubation period at room temperature they were incubated for 24 hrs at 37°C. The test agent was tested with and without S-9 at six doses. After 24 hrs the plates were analyzed for toxicity. The zone of inhibition was estimated to the nearest millimeter with a hand held ruler. Any visible zone of inhibition was considered an indication of a toxic response. With toxic agents, the high dose was chosen for the mutagenicity test that caused a slight (≤1 mm) zone of inhibition (Table 1).

g. Mutagenesis Assay: The assay was conducted March 6 and 17, 1985 using the methods of Ames et al as described in Mut. Res. 31:347-364, 1975 and in their methods update issued in May 1980. The detailed procedures are outlined in IITRI SOP No. MB53. Briefly, 0.1 ml (10⁸ cfu) of an 16-18 hr culture

of the tester strain was added to 2 ml top agar (45°C) in sterile, unused 16x150 mm glass test tubes. This was followed by addition of 0.1 ml of the test article and 0.5 ml of the S-9 reaction mixture or test article alone for the non-S-9 treated cultures. The tubes were vortex mixed then poured over minimal agar plates. Triplicate plates were made per dose for five doses. Control plates containing only the bacterial strains were also made to determine spontaneous reversion rates as well as control plates to test for sterility of each of the systems components. The plates were incubated in the dark at 37°C in an inverted position for 2 days after which they were evaluated for numbers of revertant colonies.

h. Colony Counting: The revertant colonies were scored with a Biotran™ II colony counter. The counter was calibrated against a calibration grid just prior to use. The counts were recorded in a log book as they were generated. Plates with fewer than 50 colonies were hand counted. All plates were incinerated after the evaluation was complete.

5. SUPERVISORY PERSONNEL INVOLVED IN THE STUDY

Peter W. Barbera
Robert R. Guerrero
James D. Fenters

Josephine M. Reed

Study Director
Program Director
Head, Toxicology and
Environmental Health
Supervisor, Quality
Assurance

6. SUMMARY OF DATA

- Table 1 - Dose range finding study for test article USA-01
- Table 2 - Mutagenicity results for positive controls
- Table 3 - Raw mutagenicity data for test article USA-01 and phosphoric acid
- Table 4 - Summary mutagenicity results for test article USA-01 and phosphoric acid
- Table 5 - Summary of background lawn toxicity results on test article USA-01 and phosphoric acid.

Table 1

DOSE RANGE FINDING STUDY FOR TEST AGENT USA-01

IITRI Project No. L06139-L001

IITRI Study No. SN81A

Test Agent	Conc. µg/plate	Inhibitory zone ^Δ with Strain TA98 (mm)		Toxicity
		NA	A	
H ₂ O	50.0	0	0	-
USA-01	5000.0	15	15	+
	2500.0	13	13	+
	1250.0	0	0	-
	500.0	0	0	-
	50.0	0	0	-
	5.0	0	0	-

NA= without S-9

A= with S-9

Δ= Average for two measurements

- = Non-toxic

+ = Toxic

+ = Slightly toxic

Table 2

MUTAGENICITY RESULTS FOR POSITIVE CONTROLS

IITRI Project No. L06139-L001

IITRI Study No. SN81A

Test Agent	Conc. ug/plate	Mutagenic Response									
		TA98		TA100		TA1535		TA1537		TA1538	
		NA	A	NA	A	NA	A	NA	A	NA	A
2NF	10.0	721								625	
		639								701	
		653 (671)	-	-	-	-	-	-	-	665 (664)	-
NAZ	10.0			874		1005					
		-	-	900	-	990	-	-	-	-	-
				915 (896)		921 (972)					
9A	100.0							2240			
		-	-	-	-	-	-	2005	-	-	-
								2119 (2121)			
2A	10.0		1621	1953		304		139		1793	
		-	1785	2004	-	290	-	147	-	1854	-
			1704 (1703)	1841 (1933)		315 (303)		150 (145)		1883 (1843)	

A = activated

NA = non-activated

() = average plate count

2NF = 2-nitrofluorene

NAZ = sodium azide

9A = 9-aminoacridine

2A = 2-anthramine

Table 3
RAW MUTAGENICITY DATA FOR TEST AGENT USA-01 AND PHOSPHORIC ACID

IITRI Project No. L06139-L001
IITRI Study No. SN81A

Test Agent	Conc. ug/plate	pH	Mutagenic Response									
			TA98		TA100		TA1535		TA1537		TA1538	
			NA	A	NA	A	NA	A	NA	A	NA	A
0	-	(Spontaneous Revertants)	23	27	164	190	16	17	7	6	24	27
			23	25	175	184	16	14	8	6	26	24
			26	24	160	201	15	16	7	7	23	26
			(24)	(25)	(166)	(192)	(16)	(16)	(7)	(6)	(24)	(26)
H ₂ O	100.0	6.2	20	24	164	180	16	16	6	8	24	24
			23	21	157	195	15	15	6	6	24	20
			24	25	170	182	15	16	5	7	23	25
			(22)	(23)	(164)	(186)	(15)	(16)	(6)	(7)	(24)	(23)
USA-01	2500.00	1.9	22	24	168	190	16	17	6	7	24	23
			21	24	157	180	16	15	4	8	23	27
			23	23	162	171	16	17	7	7	25	26
			(22)	(24)	(162)	(180)	(16)	(16)	(6)	(7)	(24)	(25)
	0.12	6.0 ^Δ	20	25	164	183	14	16	7	8	23	24
			23	20	150	172	16	18	6	8	25	28
			24	23	151	165	14	15	5	8	26	25
			(22)	(23)	(155)	(173)	(15)	(16)	(6)	(8)	(25)	(26)
	0.12	7.0 ⁺	21	22	180	169	14	15	7	6	25	29
			22	25	164	183	15	19	5	6	28	25
			21	23	167	190	18	16	5	9	24	25
			(21)	(22)	(164)	(177)	(16)	(17)	(6)	(7)	(26)	(26)
H ₃ PO ₄	1180.0	1.9	23	21	159	180	16	15	7	7	23	26
			21	24	163	171	15	17	6	9	26	26
			21	22	170	181	17	17	7	9	24	26
			(22)	(22)	(164)	(177)	(16)	(16)	(7)	(8)	(24)	(26)
	1050.0	7.0 ⁺	23	24	160	174	17	20	6	7	22	29
			20	24	153	167	28	16	5	7	25	25
			21	26	155	187	17	18	6	7	20	24
			(21)	(25)	(156)	(176)	(17)	(18)	(6)	(7)	(22)	(26)

continued.....

Table 3 (continued)

RAW MUTAGENICITY DATA FOR TEST AGENT USA-01 AND PHOSPHORIC ACID

IITRI Project No. L06139-L001
IITRI Study No. SN81A

Test Agent	Conc. ug/plate	pH	Mutagenic Response									
			TA98		TA100		TA1535		TA1537		TA1538	
			NA	A	NA	A	NA	A	NA	A	NA	A
H ₃ PO ₄	500.0	7.0 [*]	22	23	165	180	15	19	8	8	20	26
			22	25	169	170	18	16	8	9	24	25
			21	22	159	185	18	17	7	7	21	24
			(22)	(23)	(164)	(178)	(17)	(17)	(8)	(8)	(22)	(25)
	0.03	6.0 ^Δ	23	20	167	184	15	13	6	7	25	23
			22	25	163	188	14	15	8	8	24	26
			22	24	171	190	15	16	6	7	23	24
			(22)	(23)	(167)	(187)	(15)	(15)	(7)	(7)	(24)	(24)
	0.03	7.0 [†]	20	21	160	182	15	17	6	8	23	25
			23	20	170	190	16	15	8	8	21	24
			20	22	162	189	16	15	6	8	22	25
			(21)	(21)	(164)	(187)	(16)	(16)	(7)	(8)	(22)	(25)

NA = without S-9

A = with S-9

() = Average

* = pH adjusted to 7 with 40% NaOH

Δ = pH adjusted by dilution to 6 with pH 6.2 distilled water

† = Same as Δ except pH adjusted from 6 to 7 with 0.1 N NaOH

Table 4

SUMMARY MUTAGENICITY RESULTS FOR TEST AGENT USA-01
AND PHOSPHORIC ACID

IITRI Project No. L06139-L001
IITRI Study No. SN81A

Test Agent	Conc. µg/plate	pH	Mutagenic Response							
			TA98		TA100		TA1535		TA1537	
			NA	A	NA	A	NA	A	NA	A
USA-01	2500.00	1.9	-	-	-	-	-	-	-	-
	0.12	6.0 Δ	-	-	-	-	-	-	-	-
	0.12	7.0 \dagger	-	-	-	-	-	-	-	-
H ₃ PO ₄	1180.00	1.9	-	-	-	-	-	-	-	-
	0.03	6.0 Δ	-	-	-	-	-	-	-	-
	0.03	7.0 \dagger	-	-	-	-	-	-	-	-
	1050.00	7.0 \ddagger	-	-	-	-	-	-	-	-
	500.00	7.0 \ddagger	-	-	-	-	-	-	-	-
H ₂ O	100.00	6.2	-	-	-	-	-	-	-	-
2NF	10.00		+	0	0	0	0	0	0	0
NAZ	10.00		0	0	+	0	+	0	0	0
9A	100.00		0	0	0	0	0	0	+	0
2A	10.00		0	+	0	+	0	+	0	+

NA = Without S-9

A = With S-9

- = Non-mutagenic

+ = Mutagenic

0 = Not tested

 \ddagger = pH adjusted to 7 with 40% NaOH Δ = pH adjusted to 6 by dilution with pH 6.2 distilled water \dagger = Same as Δ except pH adjusted from 6 to 7 with 0.1 N NaOH

2NF 2-nitrofluorene

NAZ Sodium Azide

9A 9-aminoacridine

2A 2-anthracene

Table 5

SUMMARY OF BACKGROUND LAWN TOXICITY RESULTS ON TEST
ARTICLE NO. USA-01 AND PHOSPHORIC ACID

IITRI Project No. L06139-L001

IITRI Study No. SN81A

Test Agent	Conc. ug/plate	pH	Reduction of Background Lawn									
			TA98		TA100		TA1535		TA1537		TA1538	
			NA	A	NA	A	NA	A	NA	A	NA	A
USA-01	2500.00	1.9	-	-	-	-	-	-	-	-	-	-
	0.12	6.0 Δ	-	-	-	-	-	-	-	-	-	-
	0.12	7.0 †	-	-	-	-	-	-	-	-	-	-
H ₃ PO ₄	1180.00	1.9	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm
	1050.00	7.0 *	-	-	-	-	-	-	-	-	-	-
	500.00	7.0 *	-	-	-	-	-	-	-	-	-	-
	0.03	6.0 Δ	-	-	-	-	-	-	-	-	-	-
	0.03	7.0 †	-	-	-	-	-	-	-	-	-	-

NA = Without S-9

A = With S-9

* = pH adjusted to 7 with 40% NaOH

 Δ = pH adjusted by dilution to pH 6 with pH 6.2 distilled water † = Same as Δ except pH adjusted from 6 to 7 with 0.1 N NaOH

- = Non-toxic

 \pm = Slightly toxic

2. CONCLUSIONS:

Toxicity: The results of the dose range finding study (Table 1) indicate that test article USA-01 was slightly toxic to the TA98 tester strain at the 2,500 ug/plate concentration and toxic at the 5000 ug/plate dose. It was not toxic at any dose below 2,500 ug/plate both with and without S-9. The 2,500 ug/plate concentration was chosen as the high dose for the mutagenicity test.

The results shown in Table 5 indicate that in the mutagenicity assay neither the test article nor the phosphoric acid controls induced a toxic response under any of the conditions tested, except for a slight reduction in background lawn caused by the 1180 ug/control plate of phosphoric acid at pH 1.9. The slight toxic response was observed in all the strains with and without S-9.

Mutagenicity: In the mutagenicity test, the responses of all the control compounds (Table 2), solvent controls and spontaneous background revertants (Table 3) were within the limits set for the test. All of the test system components also tested sterile. The assay was therefore judged valid.

Table 4 shows a summary of the mutagenicity results for all the parameters tested in this study. The results show that neither test article USA-01 nor phosphoric acid were mutagenic under any conditions even those that maximized conditions for a positive response, i.e. high concentration (2,500 ug/plate) and low pH (1.9) for USA-01 and 1,180 ug/plate and pH 1.9 for phosphoric acid. The negative response observed with the phosphoric acid samples neutralized to pH 7 with NaOH also indicate that high osmolality caused by addition of increased amounts of sodium does not induce a false positive response in the Ames test. Treatment with S-9 did not alter the mutagenic nor the toxic response. This suggests that USA-01 does not contain promutagenic components nor toxic detoxification products.

In light of the overall negative mutagenic response regardless of pH or concentration considerations, the conclusion is that test article USA-01 is not mutagenic in the Ames test and that the false positive results reported in mammalian cell systems due to low pH or high osmolality culture conditions do not appear to apply to the Ames test.

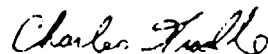
8. SIGNATURES OF SCIENTISTS INVOLVED IN THE STUDY

Peter W. Barbera
Study Director



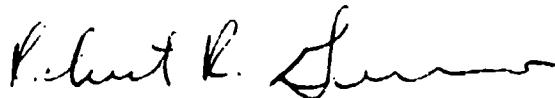
Date 6/12/85

Charles Gradle
Assistant



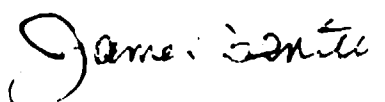
Date 6/12/85

Robert R. Guerrero, Ph.D.
Program Director



Date 6/12/85

James D. Fenters, Ph.D.
Head, Toxicology and
Environmental Health



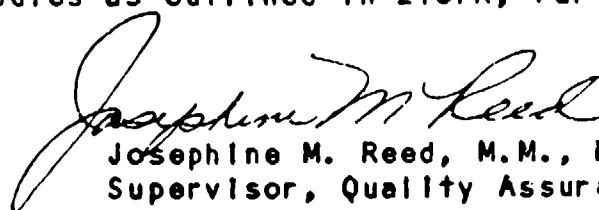
Date 6/12/85

9. STORAGE OF DATA AND REPORTS:

All raw data generated during the course of the study were retained in the IITRI Life Sciences archives as specified by government regulations. The original final report and six copies was submitted to the sponsor and one copy of the report was retained in the IITRI archives, one in the Department L files and one by the program director.

10. QUALITY ASSURANCE STATEMENT:

Laboratory operations were inspected on March 5, 6, 8, 12, 26, 27 and 29, 1985. The final draft report was audited on April 19 and 22 and May 31, 1985. Inspections and audits were performed by Josephine M. Reed. Laboratory operations conformed with IITRI Life Sciences quality assurance criteria and with GLP requirements for nonclinical laboratory studies as outlined in 21CFR, Part 58.



Josephine M. Reed, M.M., M.S.
Supervisor, Quality Assurance

FINAL REPORT

CHROMOSOME ABERRATION ANALYSIS OF AN EXTRACT
OF RED PHOSPHORUS/BUTYL RUBBER AEROSOL CONDENSATE

IITRI Project No. LO6139-L001
Study No. SN81-ABER TEST

July 1985

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1. Introduction

The purpose of this study was to evaluate the genotoxic properties of an extract from red phosphorus/butyl rubber condensate utilizing the Chinese Hamster Ovary Cell (CHO)/Chromosome Aberration Analysis In vitro assay and to determine the influence of pH on the genotoxic response.

Red phosphorus/butyl rubber (RB/BR) aerosol condensate is approximately 95% phosphoric acid; therefore, addition of even small amounts of the agent to the cell cultures constitutes a severe pH challenge.

In a recent workshop on the effects of low pH on genotoxicity in In vitro systems (EMS Workshop, Las Vegas, Nevada, February 1985), it was reported that low pH can induce false positive results. To test and control for this phenomenon, the RP/BR test article was tested at pH 6.0, 6.5 and 7.2 with concurrent phosphoric acid pH controls for the three doses. In this study, both the test article and the phosphoric acid controls were diluted with McCoy's 5A Medium Complete (3% FCS, 100 IU penicillin, 100 ug/ml streptomycin, 25 mM L-glutamine and 3 mM Hepes) to achieve the desired pH.

2. Statistical Methods Used:

Twenty-five metaphases per duplicate cultures were scored, for a total of fifty metaphases per dose. Chromosome and chromatid aberrations were converted to a "breaks/cell" value reflecting the actual number of breaks that occurred. Gaps were

converted to a gaps/cell value and are presented as a separate value. Other types of aberrations which are not easily converted to a breaks/cell value, such as, endoreduplication, aneuploidy and pulverization are presented as a percentage of aberrant cells out of the 50 scored. Means and standard deviations of the means were calculated for breaks/cell and gaps/cell.

A two-factor fixed-effects analysis of variance (ANOVA) was used to determine the effects of compound (USA-01 and H_3PO_4) and pH (pH 6.0, 6.5 and 7.2) on the number of breaks/cell and gaps/cell. The data were log-transformed prior to analysis to better approximate the assumed normality of the statistical procedure. A $p \leq 0.05$ was considered significant. Although the ANOVA was used for the overall analysis, we acknowledge that the data are not normally distributed. Therefore, the post hoc comparisons used were Poisson t tests. The post hoc comparisons evaluated the significance of observed differences between two Poisson variables using the method of E.S. Pearson and H.O. Hartley (Biometrika Tables for Statisticians, Vol.1, Table 36, London, Cambridge University Press, 1954). A $p \leq 0.05$ was considered significant.

3. Test Article and Control Information

o Test Article

a. Preparation: USA-01 RP/BR was generated by burning RP/BR in a hydraulic driven extrusion-combustion generator on Feb. 12 and 13, 1985. The extrusion/burning rate was adjusted to produce a final concentration of 1 mg/l inside a 1 m^3 stainless steel inhalation chamber. The aerosol was collected

by condensation in liquid oxygen cooled Dewar traps from 9×10^3 liters of aerosol. The sampling rate was approximately 10 liters/min for a residence time in the traps of 30 seconds. The traps were opened and the inner walls were rinsed with 30 ml of methylene chloride (MC). The traps were drained into a 1 liter glass separatory funnel and the MC and the aqueous fractions were separated and transferred to 200 ml graduated cylinders. This procedure resulted in approximately a 150 ml MC fraction and 105 ml aqueous fraction. The MC was driven off to dryness with a stream of dry nitrogen. The resultant yellow residue was dissolved in 2 ml DMSO.

The aqueous phase was transferred to a 150 ml round bottom flask. The aqueous sample was concentrated over a 2-day period to approximately 7ml with vacuum pressure delivered by a mechanical vacuum pump equipped with a liquid nitrogen cold trap. A 0.0304 g sample of the viscous yellowish-brown liquid was diluted to 100 ml with distilled water and three aliquots were analyzed for phosphate content.

For genotoxicity testing, the remaining aqueous fraction (5.64 g) was combined with the 2 ml of DMSO MC extract and the mixture diluted to 14 ml with ASTM type 1 water. The dilution was made to provide enough sample for three in vitro assays. The test article was stored at room temperature, protected from light in a sterile pyrex glass tube.

b. Physical Characteristics: The test article was a clear amber colored non-viscous liquid comprised of about 95% phosphoric acid by weight and had a density of 1.16 g/cc.

o Test Control Substances

<u>Control Agents</u>	<u>Compound</u>	<u>Source</u>	<u>Concentration</u>
Medium	McCoy's 5A Modified	MA Bioproducts 12-688B	100% v/v
Positive (+S9)	Cyclophosphamide	SIGMA C-0768	2.8 ug/ml
Positive(-S9)	Ethylmethanesulfonate	SIGMA M-0880	200 ug/ml
pH 6	Phosphoric acid	Baker 1-0260	4.4 mg/ml
pH 6.5	Phosphoric acid	Baker 1-0260	2.8 mg/ml
pH 7.2	Phosphoric acid	Baker 1-0260	1.0 mg/ml

4. Methods

The assay was performed following IITRI Chromosome Aberration Assay SOP No. MBGT-8. The methods conform to the recommendations of EPA Gene-Tox Program as outlined in Latt et al., Mut. Res. 87:17-62, 1981.

o Dosage Preparation: Dilutions of all components, including the positive controls, test agents, pH controls and cell control were prepared in unused pyrex glass tubes. The test article was prepared on a wt/volume basis. All test and control substances were prepared on the day of testing and were blended by vortex mixing for 1 min. For this study, dilutions of all compounds were prepared in McCoy's 5A Medium Complete with 3% FCS.

o S-9 Homogenate: A 9,000 x g microsomal supernate isolated from livers of adult male Fischer 344 rats induced with Aroclor 1254 was used to activate promutagens. The S-9 was prepared as per Ames et al. Mutation Research 31:347-364, 1975 and was purchased from MA Bioproducts, Walkersville, Maryland (Batch No. R137).

o Toxicity Testing: Percent viability with and without rat liver S-9 was performed to establish the dosage range for the aberration assay. The test article was tested at the following concentrations: 100 mg/ml, 10 mg/ml, and 1 mg/ml.

o Chromosome Aberration Assay: Exponentially growing CHO-K1 cells, obtained from the American Type Culture Collection in April 1982, were treated on April 4, 1985 with the test article at three concentrations (4.4 mg/ml, 2.8 mg/ml and 1.0 mg/ml) in duplicate cultures with corresponding pH values of 6.0, 6.5 and 7.2, respectively. In addition, phosphoric acid pH control cultures were run simultaneously at the same weight and corresponding pH values as those of the USA-01 treated cultures. The cells were in contact with the test agent for 12 hours (one time frame continuous exposure). The cells were arrested in metaphase with 0.05 ug/ml colcemid during the last 2 hours of incubation, then processed for aberration analysis. Twenty-five metaphases from duplicate cultures were scored for a total of fifty metaphases per dose.

o Cell Preparation for Aberration Analysis: The method used is detailed in IITRI SOP MBGT-8. Briefly, cells arrested in metaphase with colcemid were jarred loose and collected in the culture medium in the morning on April 5, 1985. The cells were pelleted with centrifugation for 7 min at 250 xg. The cells were swollen by resuspension in 0.075 M potassium chloride while incubated for 30 min at 37°C in a water bath. The cells were pelleted and fixed by resuspension in chilled Carnoy's fixative (3 parts methanol:1 part glacial acetic acid). The fixation procedure was repeated two more times and the cells resuspended in 0.5-1.0 ml of Carnoy's. Two drops of the suspension were dropped onto clean, chilled microscope slides with a disposable polypropylene pipet. The slides were air dried, then stained with 3% Giemsa in 1/15 M Sorenson's buffer.

o Scoring Aberrations: Aberrations were scored with 100X oil immersion bright field optics from 25 metaphases per duplicate culture. Aberrations were classified as chromatid, chromosome or "other" types of aberrations. Chromatid aberrations were subdivided into; chromatid breaks and gaps, isochromatid breaks and gaps and exchanges. Chromosome aberrations were subdivided into dicentrics, rings and acentric fragments. "Other" clastogenic damage categories were endoreduplication, pulverized chromosomes and sticky chromosomes. Endoreduplication, pulverized and sticky chromosomes were scored separately and presented as % of the total scored. The

aberrations were converted to a breaks/cell value using the following criteria:

<u>Aberration*</u>	<u>Abbreviation</u>	<u>Breaks/aberration</u>
<u>Chromatid Aberrations:</u>		
chromatid break	cdb	1
iso-chromatid break	icdb	2
chromatid gap	cdg	0
iso-chromatid gap	icdg	0
exchange	ex	2

Chromosome Aberrations:

Dicentric	D	2
Ring	R	2
Acentric Fragment	F	1

Other Aberrations:

Endoreduplication	e	0
Pulverized chromosomes	p	0
Sticky chromosomes	s	0

* See Appendix 1 for pictorial description of scoring system used. Breaks and gaps were scored separately and presented as a mean number/cell \pm Standard Deviation.

o Interpretation of Data: The evaluation of genotoxic potential was based on the following criteria:

1. Negative: No significant main effect or interaction ($p > 0.05$) based on ANOVA comparison of data or no significant increase in aberrations over background in post hoc comparisons in the presence of significant ANOVA results.
2. Weak clastogen: Significant main effect or interaction ($p \leq 0.05$) based on ANOVA comparisons of data. Significant aberration increase over

background ($p \leq 0.05$) based on post hoc Poisson t tests, but not twice background. These agents are highly suspect and would require further testing.

3. Strong clastogen; Significant main effect or interaction ($p \leq 0.05$) based on ANOVA comparisons of data. A $\geq 2X$ increase in chromosome aberrations in at least one dose or there is a three point dose response curve over background with at least one dose with a $p \leq 0.001$ based on post hoc Poisson t tests. These agents are considered strongly clastogenic.

5. Supervisory Personnel Involved In the Study:

Julia Harrington	Study Director
Robert R. Guerrero	Program Director
James D. Fenters	Head, Microbiology and Environmental Toxicology
Josephine M. Reed	Supervisor, Quality Assurance

6. Summary and Analysis of Data:

- o Table 1 - Toxicity Results with S-9
- o Table 2 - Toxicity Results without S-9
- o Table 3 - Chromosome Aberration Results with S-9
- o Table 4 - Chromosome Aberration Results without S-9

TABLE 1

TOXICITY TEST WITH S-9

USA-01 (mg/ml)	Total Cells (10x1 mm ²)	Viable ^Δ cells	Viability %	Avg. % Viability	% of Control*
0	78	68	87	87	100
	132	117	89		
	127	107	84		
100.0	57	55	96	92	106
	59	50	85		
	53	50	94		
10.0	58	55	95	92	106
	55	49	89		
	-	-	-		
1.0	61	61	100	99	114
	51	49	96		
	54	54	100		

^Δ $\frac{\text{Avg. viable cells treated}}{\text{Avg. viable cells control}} \times 100$

* $\frac{\text{Avg. \% treated}}{\text{Avg. \% control}} \times 100$

TABLE 2

TOXICITY TEST WITHOUT S-9

USA-01 (mg/ml)	Total cells (10x1 mm ²)	Viable ^Δ cells	Viability %	Avg. % Viability	% of Control*
0	133	123	92	90	100
	158	140	89		
	122	108	89		
100.0	-	-	-	9.5	11
	40	4	10		
	32	3	9		
10.0	36	35	97	72	80
	43	39	91		
	59	16	27		
1.0	-	-	-	92	102
	58	52	90		
	102	95	93		

Δ $\frac{\text{Avg. viable cells treated}}{\text{Avg. viable cells control}} \times 100$

* $\frac{\text{Avg. \% treated}}{\text{Avg. \% control}} \times 100$

TABLE 3

CHROMOSOME ABERRATION RESULTS ON TEST ARTICLE NO. USA-01 WITH S-9

Test Article	Dose mg/ml	pH	Tot. Cells Scored	Avg. Breaks/Cell \pm SD	Avg. Gaps/Cell \pm SD	cdg	cdb	icdg	icdb	ex	DRF	Other e p s	Total Breaks	Total Gaps
USA-01	4.4	6.0	50		1.10 \pm 1.72 *	45	11	5	0	6	0	0	0	55
	2.8	6.5	50	0.25	1.14 \pm 1.41 **	31	8	13	0	4	3	0	1	57
	1.0	7.2	50	0.24 \pm 0.	1.14 \pm 0.77 *	11	0	3	0	2	4	0	0	17
H ₃ PO ₄	4.4	6.0	50	0.28 \pm 0.64	0.88 \pm 1.48 *	23	8	11	2	1	0	0	0	45
	2.8	6.5	50	0.22 \pm 0.62	0.22 \pm 0.51	7	1	2	1	4	0	0	0	11
	1.0	7.2	50	0.20 \pm 0.53	0.10 \pm 0.39	9	4	0	0	1	2	0	0	10
Medium	0	7.2	50	0.10 \pm 0.51	0.12 \pm 0.59	2	1	2	0	2	0	0	0	5
Cp†	+	7.2	50	0.74 \pm 1.64	0.34 \pm 0.72	10	7	3	3	6	6	0	0	37

cdg chromocid gap, cdb chromatid break, icdg isochromatid gap, icdb isochromatid break

D dicentric, R ring, F fragment, e endoreduplication, p pulverized chromosomes

s sticky chromosomes

+ Cyclophosphamide (2.8 ug/ml)

* Significantly different from medium control (p<0.05) in post hoc Poisson t tests in the presence of significant ANOVA effects.

† Significant difference (p<0.05) between USA-01 and H₃PO₄ at same pH.

TABLE 4
CHROMOSOME ABERRATION RESULTS ON TEST ARTICLE USA-01 WITHOUT S-9

Test Article	Dose mg/ml	pH	Tot. Cells Scored	Avg. Breaks/Cell \pm SD	Avg. Gaps/Cell \pm SD	cdg	cdb	icdg	icdb	Frequency ex D	R F	Other e p s	Total Breaks	Total Gaps
USA-01	4.4	6.0	50	0.64 \pm 1.12 *	0.96 \pm 1.83 **	16	12	11	0	5	4	0	0	38
	2.8	6.5	50	0.34 \pm 0.92 *	0.32 \pm 1.00	6	3	5	0	6	1	0	0	16
	1.0	7.2	50	0.22 \pm 0.82	0.22 \pm 0.51	9	3	1	0	4	0	0	0	11
H ₃ PO ₄	4.4	6.0	50	0.96 \pm 1.53 *	2.22 \pm 3.01 **†	27	12	42	1	15	2	0	0	111
	2.8	6.5	50	0.34 \pm 0.77 *	0.18 \pm 0.44	7	3	1	1	4	2	0	0	9
	1.0	7.2	50	0.18 \pm 0.52	0.36 \pm 0.66	18	3	0	1	1	1	0	0	18
Medium	0	7.2	75	0.08 \pm 0.36	0.09 \pm 0.41	1	2	3	1	1	0	0	0	7
EMS†	+	7.2	50	5.68 \pm 4.29	3.06 \pm 2.79	133	96	10	3	82	3	1	0	284

cdg chromatid gap, cdb chromatid break, icdg isochromatid gap, icdb isochromatid break
D dicentric, R ring, F fragment, e endoreduplication, p pulverized chromosomes

s sticky chromosomes

+ Cyclophosphamide (2.8 ug/ml)

* Significantly different from medium control ($p < 0.05$) in post hoc Poisson t tests in the presence of significant ANOVA effects.

† Significant difference ($p < 0.05$) between USA-01 and H₃PO₄ at same pH.

7. Discussion and Conclusions

o Toxicity Results: The data in Tables 1 and 3 suggest that the RP/BR extract was not toxic at any of the concentrations tested with S-9, i.e. 114, 106 and 106% of control at the 1, 10 and 100 mg/ml concentrations, respectively. However, it caused a dose related toxic response when tested without S-9, i.e. 102, 80 and 11% of controls at the same concentrations. The data are deceptive in that although the cells were viable by trypan blue exclusion measurements, the mitotic activity was severely inhibited by the 10 and 100 mg/ml exposures. The cells exposed to the 100 mg/ml dose also appeared rounded up yet firmly attached to the plastic surfaces to the extent that it was impossible to remove them with 0.05% trypsin despite repeated attempts. These results suggest that the low pH resulting from addition of 10 (pH 2.8) and 100 (pH 1.6) mg/ml test article in culture medium has a profound effect on the cell membrane characteristics and/or on the culture vessel's growing surfaces. The cells exposed to 1.0 mg/ml (pH 7.2) concentration appeared normal.

Attempts to raise the pH of the test agent or 95% H_3PO_4 to 7.2 with 40% sodium hydroxide or with heavily buffered medium was unsuccessful because the resultant high osmolality in the culture medium caused the cells to detach from the growing surfaces. Detachment was presumably due to changes in cell membrane and/or growing surface

characteristics.

As a result of the severe technical problems caused by the low pH exposures and because of a subsequent report that low pH can result in false positive cytogenetic results in mammalian cells, the decision was made to test the test article at pH 6.0, 6.5 and 7.2 with the pH obtained by dilution with complete culture medium. The resultant concentrations were 4.4, 2.8 and 1.0 mg/ml, respectively. Concurrent phosphoric acid controls were run at the same pH and mg/ml concentration. The results of studies from two protocols are thus being presented in a single report.

o Aberration Results:

The aberration frequencies are listed in Tables 3 and 4. The results indicate that predominately chromatid type aberrations were formed following the exposure. This implies that most of the clastogenic damage occurred during "Late S" and "G₂" phases of the cell cycle. In addition, use of S-9 did not appear to alter the overall pattern of aberration frequencies.

While the means for the S-9 treated groups show a general increase in breaks and gaps with dose for both the USA-01 and H₃PO₄ exposures (Table 3), the ANOVA comparisons indicate that for breaks there was no significant main effect due to

compound ($p=0.325$), dose ($p=0.094$), or compound by dose ($p=0.625$). Therefore, post hoc comparisons were not performed.

For gaps, there were significant ANOVA results for compound ($p=0.002$), dose ($P=0.001$) and the compound by dose interaction ($p=0.017$). Post hoc comparisons indicate that all three concentrations of test article USA-01, pH 6.0 (4.4 mg/ml), pH 6.5 (2.8 mg/ml), and pH 7.2 (1.0 mg/ml), and the top dose for H_3PO_4 , pH 6.0 (4.4 mg/ml), were significantly different from the medium control ($p \leq 0.05$). However, the comparisons between the USA-01 groups and corresponding pH groups for H_3PO_4 indicate that only the pH 6.5 (2.8 mg/ml) result was significantly different ($p \leq 0.05$) and the difference was due to the higher mean for the USA-01 group.

For the non-S-9 treated groups (Table 4), the means for breaks and gaps also showed a general dose responsive increase for both the USA-01 and H_3PO_4 exposures. The ANOVA indicated that for breaks, there was a significant effect for dose ($p=0.001$), but not for compound ($p=0.477$) or the compound by dose interaction ($p=0.681$). For gaps, there were significant results for all three comparisons (compound, $p=0.018$; dose, $p=0.001$, and compound by dose, $p=0.004$).

Post hoc comparisons indicate that for breaks, only the USA-01 pH 6.0 (4.4 mg/ml) and pH 6.5 (2.8 mg/ml) doses and the pH 6.0 (4.4 mg/ml) H_3PO_4 dose were significantly different ($p \leq 0.05$) from the medium control. However, there was

no significant difference between the USA-01 and corresponding pH levels of H_3PO_4 ($p > 0.05$).

For gaps, the ~~post hoc~~ tests indicate that only the USA-01 pH 6.0 and the H_3PO_4 pH 6.0 doses were significantly different ($p \leq 0.05$) from the medium control and from each other and the difference was due to the higher mean for the H_3PO_4 group.

The conclusion is, therefore, that while dose responsive increases in breaks were observed, the increases were not significant when compensation was made for the pH effect. The increase in breaks caused by the test article USA-01 is a pH-induced phenomenon caused by the high concentration of phosphoric acid in the same (95%) and not by other clastogenic components in the sample. Further, while gaps also increased with dose, correction for pH effects also negated increases in gaps due to USA-01 with the exception of the pH 6.5 (2.8 mg/ml) dose in the S-9 treated group.

Gaps are controversial lesions. They are by definition not breaks; however, they are useful as indicators of trends or patterns in clastogenic responses since, in general, increases in both gaps and breaks are seen following exposure to clastogenic agents. Since in this study, with the one exception, the increase in gaps also appeared to be pH related, the conclusion is that the positive gap results in the one instance was an isolated event and, in the face of the

negative break results, should not change the ultimate conclusion that test article USA-01 is non-clastogenic to CHO cells at the concentrations tested.

The possibility remains, however, that the 4.4 mg/ml high dose used in this study was below the level of sensitivity for the system to detect gross clastogenic damage and that testing at higher concentrations would give a positive response. However, testing higher concentrations was impossible due to the prohibitively low pH conditions that resulted in the culture medium when higher concentrations of the test article were used.

8. Signature of Scientists Involved in the Study:

Julie Harrington
Study Director

Julie Harrington

Date 7/2/85

Robert R. Guerrero
Program Director

Robert R. Guerrero

Date 6/20/85

James D. Fenters
Head, Microbiology and
Environmental Toxicology

James Fenters

Date 7/2/85

Josephine M. Reed
Supervisor, Quality Assurance

Josephine M. Reed

Date 7/2/85

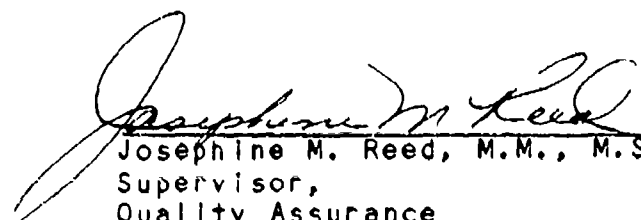
9. Storage of Data and Reports:

All raw data generated during the course of the study were retained in the IITRI Life Sciences archives as specified by government regulations. The original final report was submitted to the sponsor and one copy of the report was retained in the IITRI archives, Division L files and one by the program director.

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10. Quality Assurance:

- o Quality Assurance Statement: Laboratory operations were inspected March 12 through 15, 1985 and April 3,4,5 and 8, 1985 by Josephine M. Reed. The final draft report was audited on June 26, to 27, 1985 by Josephine M. Reed.


Josephine M. Reed, M.M., M.S.
Supervisor,
Quality Assurance

11. Appendix - Pictorial interpretation of "breaks" scoring system used.

Category	Interphase	Metaphase	Interpretation	Symbol	Breaks
<u>Chromosome-type Aberrations (G₁ or early S)</u>					
<u>Break</u>			1 Acentric frag.	F	1
<u>Exchanges</u>					
• Interchromosomal					
		a.	a. 1 Dicentric + 1 acentric frag.	D	2
		b.	b. 1 Dicentric + 2 acentric frag.	D	2
• Intrachromosomal					
			1 Ring + 2 acentric frag.	R	2
<u>Chromatid-type Aberrations (late S or G₂)</u>					
<u>Break</u>		a.	a. 1 break	cdb	1
		b.	b. 4 breaks	4 cdb	4
		c.	c. isochromatid break	icdb	2
<u>Exchanges</u>					
• Interchromosomal					
		a.	a. 2 exchange	2 ex	4
		b.	b. 1 exchange + acentric frag.	ex	2
• Intrachromosomal					
		a.	a. 1 exchange	ex	2
		b.	b. 1 chromatid ring + frag.	R	2

FINAL REPORT

DNA REPAIR ASSAY IN PRIMARY RAT HEPATOCYTES ON AN EXTRACT
OF RED PHOSPHORUS/BUTYL RUBBER AEROSOL CONDENSATE

IITRI Project No. LO6139L001
Study No. 81-DNA-1

July 1985

IIT RESEARCH INSTITUTE

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1. PURPOSE:

The purpose of this study was to assess the mutagenic properties of an extract of red phosphorus/butyl rubber condensate and to determine the influence of pH on the mutagenic response.

Red phosphorus butyl rubber (RP/BR) aerosol condensate is about 95% phosphoric acid. Therefore, addition of even small amounts of the agent constitutes a severe pH challenge to the cells.

In a recent workshop on the effects of low pH and high salt concentrations on genotoxicity in vitro (EMS workshop, Las Vegas, Nevada, February 1985), it was reported that low pH and high osmolality conditions can induce false positive results in in vitro systems. Subsequently the RP/BR test article was tested at pH 6.0 and 7.0 with phosphoric acid pH controls for the two doses. The USA01 (RP/BR) sample and the H_3PO_4 controls were diluted with Williams E medium, pH 7.2, to achieve pH 6.0.

2. STATISTICAL METHODS USED:

Fifty interphase nuclei were evaluated per dose. Mean net grain counts and standard deviation of the mean were calculated for each dose. A test agent was considered genotoxic if it induced a net grain count of 5 or more per nuclei in triplicate coverslips when tested within a nontoxic range (>10% survival).

3. TEST ARTICLE AND CONTROL INFORMATION:

o Test article:

a) Physical Characteristics: The test article USA01, RP/BR was generated by burning RP/BR in a hydraulic driven extrusion-combustion generator on February 12 and 13, 1985. The extrusion/burning rate was adjusted to a final concentration of approximately 1 mg/l inside a 1 m³ stainless steel inhalation chamber. The aerosol was collected by condensation in liquid oxygen cooled Dewar traps from 9×10^3 liters of aerosol. The sampling rate was approximately 10 liters/min for a residence time in the traps of 30 seconds. The traps were opened and the inner walls were rinsed with 30 ml of methylene chloride (MC). The traps were drained into a 1 liter glass separatory funnel and the MC and the aqueous fractions were separated and transferred to 200 ml graduated cylinders. This procedure resulted in approximately 150 ml MC fraction and 105 ml aqueous fraction. The MC was driven off to dryness with a stream of dry nitrogen. The resultant yellow residue was dissolved in 2 ml of DMSO.

The aqueous phase was transferred to a 150 ml round bottom flask. The aqueous sample was concentrated over a 2-day period to 7 ml with vacuum pressure delivered by a mechanical vacuum pump equipped with a liquid nitrogen cold trap. A 0.0304 g sample of the viscous yellowish-brown liquid was diluted to 100 ml with distilled water and three aliquots were analyzed for phosphate content. The resultant phosphoric acid concentration was determined to be 90.6% by weight.

For genotoxicity testing, the remaining aqueous fraction (5.64g) was combined with the 2 ml of DMSO MC extract and the mixture was diluted to 14 ml with ASTM type 1 water on February 19, 1985. The dilution was made to provide enough sample for three *in vitro* assays. The test article was stored at room temperature protected from light in a sterile pyrex glass tube. The density of the test article was 1.16 g/ml.

b) Solvent: Concentrations of the test article and the air sample were prepared in Williams E medium containing 10 uCi/ml of tritiated thymidine.

c) Dosage: For the test article, concentrations of 1.000 mg/ml, 0.333 mg/ml and 0.033 mg/ml were prepared with a final pH of 6.0, 7.0 and 7.0 respectively. Two dilutions of phosphoric acid, with final concentrations of 1.000 mg/ml and 0.333 mg/ml and a final pH of 6.0 and 7.0, respectively, were also prepared. The air sample was diluted to give an approximately 10% solution.

o Control Substances:

<u>Control</u>	<u>Compound</u>	<u>Source</u>	<u>Conc.</u>
Positive	2-acetylamino-fluorene	Tridom-Fluka	100 nMol/ml
Negative	Biphenyl	Aldrich Chemical Co.	100 nMol/ml
Cell	WME*	Gibco	100%

* Williams medium + 20 mM glutamine + 50 ug/ml gentamycin

4. METHODS:

The assay was performed following procedures outlined in IITRI DNA Repair SOP No. MBGT-2. The methods are based on those outlined by Williams in Cancer Res. 37:1845-1851, 1977 and include the improvements made by Williams in Cancer Letters 4:69-75, 1978 and by Williams et al in *In Vitro* 13:809-817, 1977.

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o Experimental Design: The test article was tested for toxicity in rat hepatocytes prior to the DNA repair assay. The toxicity results were used to establish the dosage range for the DNA repair assay. In the repair assay, hepatocytes on coverslips were exposed for 18-20 hr to 5 concentrations in triplicate of the test agent in the presence of tritiated thymidine ($^3\text{H-TdR}$). DNA damage caused by the exposure and repaired by unscheduled DNA synthesis was measured with autoradiography. Silver grains deposited over interphase nuclei, indicating sites of DNA repair, were counted in 50 nuclei. The counts were also corrected for nonspecific binding of $^3\text{H-TdR}$. A net grain count of 5 or more was considered a positive response.

o Preparation of Hepatocytes: A F344 Fisher male rat (Harlan-Sprague Dawley) was anesthetized with 50 mg/kg nembutal. The liver was surgically exposed then perfused in situ with 0.5 M ethylene glycol-bis-(B-aminoethylether) N-N'-tetracetic acid (EGTA) followed by 100 u/ml collagenase, type 1 (Sigma). The liver was aseptically removed and placed in a sterile petri dish. In a laminar flow hood, the outer capsule was removed from the liver and the hepatocytes were gently separated from the organ. The cells were then collected in 37°C WMES medium and assayed for density and viability.

o Toxicity Testing: One million viable hepatocytes were seeded into T-25 flasks. Three flasks were prepared for each of five dilutions of the test article. The highest concentration tested did not exceed 10% weight per volume. The cells were allowed to attach for 2 hr and then were exposed to the test article for 18-20 hr at 37°C on February 19, 1985. Trypan blue was added (0.15 ml of a 0.4% solution) to each flask. After 5 min, the cells were rinsed then fixed with 1 ml of 5% formalin. The cells were evaluated for toxicity using dye exclusion and cell number as endpoints. The determination was made by calculating the ratio of viable cells in 10 randomly selected 1 mm² areas of each flask utilizing a 1 mm² grid in the eyepiece of an inverted microscope.

o DNA Repair Testing: On May 29, 1985, one million viable hepatocytes were seeded onto 25 mm Thermanox coverslips (M.A. Bioproducts) housed in 34 mm 6-well tissue culture cluster dishes (Costar). The cells were allowed to attach for 2 hr then were rinsed with WME medium and treated with five concentrations of the test agent in triplicate, including a toxic dose when possible. The medium also contained 10 mCi/ml $^3\text{H-TdR}$ (60-80 Ci/mMole specific activity). Positive, negative, solvent and cell controls were tested in parallel. The cultures were incubated for 18-20 hrs at 37°C in a humidified 5% CO₂ incubator.

o Coverslip Preparation: Following the exposure, the coverslips were rinsed with three changes of WME medium, then the cells were swollen in 1% sodium citrate for 15 min. The cells were fixed in three changes of Carnoy's fixative (3 parts ethanol:1 part glacial acetic acid). The coverslips were air dried, then attached to glass slides, cell surface up, with permount mounting medium.

o Autoradiography: On May 31, 1985, the slides were dipped in Kodak NTB emulsion. They were air dried overnight, then the slides were placed in light tight slide boxes for 10 days at 45°C. The slides were developed with Kodak D-19 developer, air dried and stained with Harris' alum hematoxylin and eosin on June 10, 1985. All procedures, up to the air drying of the slides, were completed in total darkness.

o Grain Counts: Grain counts were made on 50 randomly selected normal nuclei per slide (3 slides/dose) with a New Brunswick Biotran Colony Counter. The counter interfaced with a TV system coupled to a compound light microscope. The counts were corrected for nonspecific ³H-binding by subtracting from the nuclear count, the highest of three counts taken from cytoplasmic areas adjacent to the nucleus. Means and standard deviations were calculated for each set of three slides.

5. DOSAGE PREPARATION AND DRUG HANDLING:

Upon receipt of all test and control materials, the date of receipt and approximate quantities were recorded. Liquid test articles were measured on a weight to volume basis and dilutions of all compounds, including the positive control and solvent controls, were prepared in glass scintillation vials. All test and control substances were freshly prepared the day of use and were blended by vortex mixing for 1 min prior to testing. The control compounds were stored according to sponsor's recommendations regarding temperature, humidity and protection from light. The highest concentration used did not exceed 10% weight per volume and the solvent did not exceed 1% volume per volume.

6. Supervisory Personnel Involved in the Study:

Kathleen V. Ketels

Study Director

Robert R. Guerrero

Program Director

James D. Fenters

Head, Toxicology and
Environmental Health

Josephine M. Reed

Quality Assurance
Supervisor

7. SUMMARY AND ANALYSIS OF DATA:

- o Table 1 - Toxicity Results
- o Table 2 - DNA Repair - Test Article and Control Results
- o Table 3 - DNA Repair - Air Sample and Control Results

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IITRI Study No. 81-DNA-1

TABLE 1

Toxicity Results on
Test Article No. USA01

Conc. mg/ml	pH	Cells/cm ²	Cell Number Cells/flask(x10 ⁴)	Avg. ^a (x10 ⁴)	Viability ^b %
100.0	<1	2674	6.7	6.4	81.3
		2615	6.5		
		2394	6.0		
50.0	1.5	3258	8.1	6.8	86.0
		1934	4.8		
		2929	7.3		
10.0	3.0	2568	6.4	5.3	67.0
		2108	5.3		
		1645	4.1		
5.0	5.5	Toxic	Toxic	Toxic	
		Toxic	Toxic		
		Toxic	Toxic		
1.0	7.0	2358	5.9	4.7	59.6
		714	1.8		
		2557	6.4		
Cell Control	7.0	3594	9.0	7.9	100.0
		3207	8.0		
		2972	7.4		
		3028	7.6		
		3196	8.0		
		2896	7.2		

a Each flask was initially seeded with 1x10⁶ cells

b % viability: $\frac{\text{Avg number of cells in test dilution}}{\text{Avg number of cells in cell control}} \times 100$

TABLE 2
DNA Repair Results on Test Article No. USA01,
Phosphoric Acid, and Control Agents

Test Sample	Control Agent	Conc. mg/ml	pH	Net Grain Counts ^b			Genotoxicity ±/±
				Mean	± SD	Avg.	
USA01		1.000	6.0	0.38	1.02	0.75	-
				1.04	2.85		
				0.84	1.93		
		0.333	7.0	2.63	4.61*	2.06	-
				2.48	4.23		
				1.20	2.60		
		0.033	7.0	1.40	3.22*	0.84	-
				0.68	2.90*		
				0.64	1.93		
Phosphoric Acid		1.000	6.0	0.88	2.14	0.65	-
				0.60	1.70		
				0.46	2.02		
		0.333	7.0	1.18	2.90	0.93	-
				0.52	1.54		
				1.08	3.06		
	Positive Control 2-AAF	0.100	ND	10.26	10.43	12.86	+
				14.68	12.12		
				13.64	15.71		
	Negative Control Biphenyl	0.200 ^e	ND	0.48	1.85	0.71	-
				0.50	1.45		
				1.14	3.06		
	Cell Control		ND	1.00	2.04	0.67	-
				0.58	1.81		
				0.42	1.40		

^a Net grain counts from 50 nuclei per coverslip

+ Positive genotoxic response

- Negative genotoxic response

SD Standard deviation

* Less than 50 nuclei counted

^e Incorrect concentration used due to dilution error

ND Not done

TABLE 3
DNA Repair Results on Air Sample^a
and Control Agents

Test Sample	Control Agent	Conc. mg/ml	Net Grain Counts ^b			Genotoxicity +/-
			Mean	± SD	Avg	
Air Sample			1.14	2.91		
			0.70	2.34	0.92*	-
	Positive Control	0.200 ^c	23.26	12.39		
	2-AAF		12.04	8.72	15.67	+
			11.70	9.04		
	Negative Control	0.100	3.30	6.43		
			1.22	3.37	2.26	-
			2.26	4.35		
	Cell Control		1.64	3.88		
			1.70	4.07	1.44	-
			0.98	2.94		

^a The Air Sample was tested in a separate assay from the other Test Samples.

^b Net grain counts from 50 nuclei per coverslip

+ Positive genotoxic response

- Negative genotoxic response

SD Standard deviation

^c Incorrect concentration used due to dilution error

* Two slides only

8. CONCLUSIONS:

o Toxicity: The data in Table 1 indicate that the RP/BR extract was toxic at the 5.0 mg/ml dose but only slightly toxic at the 1.0 mg/ml and all doses above 5.0 mg/ml i.e. % viability was 59.6, 0.0, 67.0, 86.0, and 81.3 for the 1.0, 5.0, 10.0, 50.0 and 100.0 mg/ml concentrations, respectively. However, while the cells exposed to concentrations higher than 5.0 mg/ml appeared viable by trypan blue exclusion, they were quite abnormal metabolically and in appearance. They were rounded up yet firmly attached to the coverslips and in a preliminary DNA repair trial run, unscheduled DNA synthesis was totally suppressed indicating that the hepatocytes were severely affected by the higher doses. These secondary toxic effects most likely reflect perturbances in cell membrane permeability and in biopathways involved in the repair process, i.e., suppression of exonuclease, endonuclease and ligase activities, caused by low pH conditions, i.e. pH 3.0, 1.5 and <1.0 for the 10.0, 50.0 and 100.0 mg/ml doses, respectively.

For the reasons stated above it was felt that 5.0 mg/ml dose reflected the top of the true toxicity range for test article USA-01 especially since the pH for that dose fell within the pH range known to be tolerated by cells in vitro (pH 5.5) and the morphology of the cells in culture looked normal. Therefore, 1 mg/ml was chosen as the top dose for the DNA repair assay.

o DNA Repair: The DNA repair results shown on Table 4 indicate that test agent USA-01 was not genotoxic at any dose tested i.e. 0.84, 2.06 and 0.75 average net grains/nucleus for the 0.033, 0.333 and 1.000 mg/ml concentrations. The phosphoric acid pH controls were also negative i.e. 0.93 and 0.65 average net grains/nucleus for pH 7.0 (0.333 mg/ml and pH 6.0 (1.000 mg/ml) concentrations, respectively.

The positive and negative controls fell within the accepted range and there was no evidence of microbial contamination, therefore, the assay was considered valid. Based on the results of this study the conclusion is that test article USA-01 does not cause an increase in DNA repair in primary hepatocytes and therefore is not genotoxic within the concentration range tested. One can't discount the possibility that higher concentrations would give a positive response. However, testing higher concentrations was not possible since they were toxic to the cells. Another conclusion is that low pH (pH 6.0) does not cause an artifactual positive response in the primary rat hepatocyte/DNA repair assay.

9. SIGNATURE OF SCIENTISTS INVOLVED IN THE STUDY:

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Study Director

Kathleen V. Ketels

Date 9/18/85

Robert R. Guerrero
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James D. Fenters
Head, Toxicology and
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James D. Fenters

Date 9/18/85

Josephine M. Reed
Quality Assurance Supervisor

Josephine M. Reed

Date 9/18/85

10. STORAGE OF DATA AND REPORTS:

All raw data generated during the course of the study will be retained in IITRI Life Sciences Archives as specified by government regulations. The original final report is submitted to the sponsor and one copy of the report will be retained in the IITRI archives, one in the Division L files and another by the program director.

11. QUALITY ASSURANCE

o Quality Assurance Statement: Laboratory operations were inspected on February 19, March 28 and 29 and May 20 and 31, 1985. The final draft report was audited on August 23, 1985. Inspections and audits were performed by Josephine M. Reed. The laboratory operations were found to conform with IITRI Life Sciences quality assurance criteria and with GLP requirements for Nonclinical Laboratory studies as outlined in 21CFR, part 58.

Josephine M. Reed
Josephine M. Reed, M.M., M.S.
Supervisor,
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